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Evaluation of the host immune response to Mycobacterium avium subsp. paratuberculosis in naturally infected periparturient dairy cows

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Evaluation of the host immune response to *Mycobacterium avium* subsp. *paratuberculosis* in naturally infected periparturient dairy cows

by

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I dedicate this dissertation to my loving husband, Darrin Karcher.

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ACKNOWLEDGEMENTS

"To get through the hardest journey we need take only one step at a time,
but we must keep on stepping." Chinese Proverb

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CHAPTER ONE

INTRODUCTION

Infections caused by the intracellular pathogen *Mycobacterium. avium* subsp. *paratuberculosis* (MAP) have a devastating impact on the dairy industry. Dairy cows generally are infected as neonates through the ingestion of water or feed contaminated with the bacteria. Animals will remain in the subclinical, or asymptomatic state, of the disease until a period of extreme stress, such as parturition. The clinical stage of the disease is characterized by severe weight loss, intermittent diarrhea, and fecal shedding of the bacteria. Currently, limited data exist on the effect of periparturient immunosuppression on the progression of Johne's disease (JD) to the clinical stages. Furthermore, a better understanding of the host immune response during this critical time period is essential for management of this and other diseases. The experiments described in the subsequent sections of this dissertation are guided by the hypothesis that the host immune response to MAP infection is altered during the periparturient period.

DISSERTATION ORGANIZATION

The objective of Experiment 1, described in Chapter 2, was to characterize cytokine gene expression and secretion in dairy cows naturally infected with MAP during the periparturient period as compared with healthy control cows. The progression of the disease is dependent upon the cytokine microenvironment. The T helper 1 (Th1), or cell-mediated,

cytokines include interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1α, and IL-12. These cytokines normally are upregulated in subclinical animals. In contrast, as the disease progresses to the clinical stage, the cytokine profile transitions from a Th1 to a Th2, or humoral, immune response. Characteristic Th2 cytokines that are upregulated in clinical animals include IL-4 and IL-10. A third subset, Th3 cells, secrete transforming growth factor (TGF)-β.

The objective of Experiment 2, described in Chapter 3, was to determine the percentages of $CD4^+$, $CD8^+$, and γδ T-cells, B-cells, and monocytes in the peripheral blood of dairy cows naturally infected with MAP during the periparturient period as compared with healthy control cows. In addition, cell populations were delineated further by staining for CD5, a marker for T- and B-cell activation. Parturition has a major impact on the number of T- and B-cells, both components of the adaptive immune system, and the number of monocyte/macrophages, effectors of the innate immune system in the peripheral blood of healthy dairy cows. This shift in Th1 to Th2 immunity is characterized by a decreased percentage of peripheral blood T-cells and an increase in the percentage of B-cells for clinically infected cows.

The objective of Experiment 3, described in Chapter 4, was to evaluate osteopontin (Opn) gene and protein expression in dairy cows naturally infected with MAP during the periparturient period as compared with healthy controls. In addition, Th1 and Th2 cytokines were evaluated for expression and secretion and correlated with that of Opn. Investigation of the role of Opn in JD is of interest based upon its ability to influence cytokine expression and to improve host defense against mycobacterial infections. Osteopontin is capable of

enhancing Th1 cytokine expression by augmenting IL-12 and subsequently inducing $CD4^+$ T-cell differentiation into Th1-like cells.

The objective of Experiment 4, described in Chapter 5, was to identify Opn in the ileum and ileocecal (IC) lymph node of dairy cows naturally infected with MAP and to compare the frequency and intensity of staining between the different infection groups. The formation of granulomas at the site of MAP infection is critical for the control of new and existing infections. Studies evaluating granuloma formation in other mycobacterial infections supported an upregulation of Opn in the inflamed tissues. Furthermore, Opn has been shown to enhance the production of IFN-γ and TNF-α, two cytokines important for the formation of granulomas.

LITERATURE REVIEW

Johne's Disease

Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is estimated to infect more than 22% of US dairy herds and cost the US dairy industry $250 million annually (Ott et al., 1999). It was first described in 1895 by Johne and Frothingham (Collins and Manning, 2007). Both noted that the tissue samples from a deceased dairy cow consisted of a thickened intestinal mucosa and enlarged mesenteric lymph nodes. It is a devastating disease that is an issue of both economics and animal welfare. Although domestic and wild ruminants primarily are infected, MAP also has been isolated from monogastric animals, such as fox, rat, badger, and crow (Beard et al., 2001). *M. paratuberculosis* is a weakly gram-positive, facultative, acid-fast bacillus. Advantages of this intracellular

bacterium include its ability to avoid antibodies, utilize complement, and utilize host nutrients. Once infected, cows generally remain in the subclinical, or asymptomatic, stage of the disease for the first few years of life and then the disease progresses to the clinical stage. The clinical stage of the disease is characterized by severe weight loss, intermittent diarrhea, and fecal shedding of MAP and may ultimately lead to death of the animal. This literature review focuses on the host immune response of periparturient dairy cows to MAP infection.

Pathogenesis of Johne's Disease

Transmission of Mycobacterium avium subsp. paratuberculosis

The transmission of MAP generally occurs by the interaction of the cow with her calf. Calves are most likely to be infected with MAP during the first six months of life (Sweeney, 1996). Compared with older adult cows, calves are more susceptible to MAP infection. When one-month-old calves were challenged with MAP, after 150 days, the calves had a greater number of MAP organisms and lesions in their tissues compared with adult cattle ranging in age from 5 to 11 years (Larsen et al., 1975). The majority of infections are caused by fecal shedding of the bacteria by clinical adult cows. Subclinically infected animals shed few, if any, bacteria in their feces. Amazingly, at 15°C, MAP can survive in cattle manure slurry for up to 98 days (Jorgensen, 1977) and transmission does not seem to be a seasonal problem. The bacteria have been reported to survive in cattle manure slurry for 21 days at 35°C (Olsen et al., 1985). In addition to fecal shedding, in clinical cows there have been reports of in utero transmission during gestation (Sweeney et al., 1992). MAP also has been isolated from the colostrum and milk of clinically infected cows (Streeter et al., 1995). In

these three scenarios, the source of MAP infection was consistently from clinically infected cows. Infected cows do not begin to shed the bacteria until an extreme period of stress, such as parturition. This shedding generally is between 2 and 3 years of age. However, more recently, Van Roermund et al., (2007) provided data to support horizontal transmission of MAP between calves suggesting that calves can shed sufficient quantities of MAP to infect other animals.

Peyer's Patches and M cells

In order to establish infection, the bacteria must be able to breach the host by establishing a route of entry into the body. Gut-associated lymphoid tissue (GALT) in the intestines is a site of host vulnerability. This area is advantageous for MAP in that it provides a source of entry. In ruminant animals, the GALT consists of aggregates of lymphoid follicles called Peyer's patches (PP). The PP extend into the intestinal lumen as dome-shaped structures consisting primarily of lymphocytes, macrophages, and dendritic cells (DCs). Although these patches are located throughout the jejunum and ileum, the ileum accounts for 80-90% of the total mass of PP tissues. Unlike other sites of the intestinal epithelium, PP do not contain mucous secreting goblet cells. This feature provides the invading bacteria with ever greater accessibility to the host.

Within the PP, the bacteria gain entry by utilizing specialized cells known as M cells. M cells are important because they are capable of sampling antigen from the lumen of the small intestine and delivering it to the cells of the immune system. MAP is translocated across the epithelium by these cells. M cells differ from other intestinal epithelial cells in that they express integrins on their luminal surface. MAP binds to M cells by forming a

fibronectin bridge between the integrins on the M cells and the fibronectin attachment proteins on MAP (Secott et al., 2004). Compared with the enterocytes of the rest of the intestine, M cells are shorter and do not have closely packed microvilli (Owen, 1999). Ileal PP are one of the largest producers of B lymphocytes in ruminant animals. Lymphocytes do not enter M cells but are able to pass between them (Owen, 1999). Another unique feature of M cells is that they do not contain lysosomes that will degrade the invading bacteria (Owen et al., 1986). This feature insures that the bacteria will be intact during transcytosis until it is released from the cell.

The ability of MAP to gain entry into the intestines via the PP and M cells is key for its initial attempts to establish infection in the host.

Immunology of Johne's Disease

Innate Immune Response

The purpose of the innate immune response is to provide an initial line of defense against invading pathogens. Once MAP has transcytosed the M cell, it will encounter subepithelial and intraepithelial macrophages (Momotani et al., 1988). There are key features of mycobacteria that provide an advantage in evading the host immune response. Mycobacteria have a rich peptidoglycan layer that contains a thick lipoarabinomannan (LAM) component. The LAM is very hydrophobic, and this trait is advantageous to the mycobacteria because it delays the formation of the phagolysosomes (Fratti et al., 2003). Under normal conditions, the macrophage can phagocytize a pathogen by opsonic (CR1 and CR2) or non-opsonic (mannose and scavenger receptors) uptake. Typically, large particles

are taken up into the phagosome. A macrophage in early activation will have Rab5 and EEA1 molecules on the phagosomal membranes and an internal pH of 6.5. Activation of the macrophage includes recruitment of vesicular ATPases to supply the protons needed to acidify the vacuole (Singh et al., 2006). A phagosome in late stage will express Rab7 and will have an internal pH of 5.5. Finally, when fusion of the phagosome and lysosome has occurred, Rab9, LAMP-1, and cathepsin D are upregulated and expressed. MAP is also capable of halting phagosome fusion by blocking the release of calcium. The pH in this environment is 4.5.

Mycobacteria have a unique ability to halt the activation of the phagosome during the early stages. The bacteria utilize the macrophages as intracellular niches to survive and replicate, essentially evading the host. In a 7-day period, MAP is able to multiply by 200-250% within monocyte-derived macrophages and monocytes (Zurbrick and Czuprynski, 1987). The ability of MAP to avoid digestion is also evident by the finding of intact bacteria in macrophages four weeks post-infection (Bendixen et al., 1981). Using the mouse macrophage cell line J774A.1, Hostetter et al. (2003) showed that in phagosomes containing live MAP there is increased expression of the early activating marker, transferrin receptor, and decreased expression of LAMP-1 compared with macrophages containing dead MAP. There is down-regulated expression of vesicular proton-adenosine triphosphatase, a necessity for proper phagosomal acidification, in MAP-infected macrophages (Sturgill-Koszycki et al., 1994). The ability to prevent the maturation of the phagosome is not unique to MAP. Other mycobacteria exhibit this similar characteristic. For example, macrophages that have been infected by *M. avium* fail to acidify below pH 6.3 (Sturgill-Koszyzki et al., 1994).

IFN-γ is produced by T cells and provides the major stimulatory signal for macrophage activation. Mycobacteria downregulate surface expression of the IFN-γ receptor (Singhal et al., 2007). When MAP infected J774A.1 macrophages are treated with IFN-γ and LPS, there is a greater ability of the phagosomal compartment to acidify and mature into functional phagolysosomes compared with the control (Hostetter et al., 2002). Activated macrophages secrete nitric oxide, reactive oxygen intermediates (H_2O_2 and OH^-) and T helper 1 (Th1) cytokines. These cytokines will be reviewed in a later section, but include IL-1, IL-12, IL-8, and TNF-α. In addition to secreting cytokines, mycobacteria block iNOS recruitment to phagosomes and subsequently reduce the secretion of nitric oxide by the macrophage (Miller et al., 2004). Receptors on macrophages important for MAP entry include CR1, CR3, CR4, FcR, mannose-receptor, LPS-binding receptor, CD14, transferring receptor, and scavenger receptors surfactant protein A receptor.

Formation of Granulomas

The formation of granulomas at the site of MAP infection is critical for the control of new infections. A hallmark characteristic of Johne's disease is the formation of these granulomas. Granulomas are inflammatory lesions that are primarily located in the GALT, lamina propria, and submucosa and are in close proximity to the lymphoid tissue of the PP. They are important for the containment of the bacteria but may also result in the destruction of tissue via the inflammatory response. Activated T lymphocytes secrete IFN-γ, which acts to recruit and activate additional macrophages to the site of infection. In addition to IFN-γ, TNF-α is also critical for the development of protective granulomas (Roach et al., 2002).

In sheep, the early subclinical stage of the disease is characterized by the formation of tuberculoid-type lesions. These lesions are not typically found in MAP-infected cows. These lesions are present in the ileal lamina propria and subcapsular sinus and are composed primarily of lymphocytes, plasma cells, and macrophages. In situ hybridization of the ileum from subclinical dairy cows revealed IL-18 (proinflammatory) mRNA present in the macrophages in the lamina propria, submucosa, and lymphoid follicles of PP (Tanaka et al., 2005). The presence of $CD4^+$ T-cells is important in the acceleration of granuloma formation. This presence directly results in the increase of TNF-α and IFN-γ production (Hansch et al., 1996). When weaned Merino lambs were infected orally with 10^7 *M. avium* subsp. *paratuberculosis*, there were increased numbers of $CD4^+$ T-cells in the ileal and jejunal PP and mesenteric lymph nodes compared with the control lambs four months after initial infection (Reddacliff et al., 2004). However, significant histological lesions were not detected in the ileal tissue of infected lambs. Furthermore, the number of resident macrophages in the ileum of MAP-infected cows is three times that of noninfected cows (Lee et al., 2001). The increase in macrophages is expected based upon the upregulation of IL-12 secretion by $CD4^+$ T-cells in the tissue. Intracellular adhesion molecule-1 (ICAM), a transmembrane glycoprotein, is important for the recruitment of macrophages to the site of infection. Challenging ICAM knock-out mice with *M. tuberculosis* resulted in extensive cellular infiltration, failure to organize granulomas to contain the bacteria, and eventual death (Saunders et al., 1999).

As the disease progresses in sheep, there is a switch in the type of lesion from tuberculoid to lepromatous. Again, these types of lesions are not typically observed in the bovine. These lesions are found in the lamina propria, submucosa, and the lymphoid follicles

of PP in the ileum. Langerhans cells infiltrate the tissue at the site of the lesion. These cells are large cells formed by the fusion of macrophages. During the clinical stage, there is a high bacterial load in the granulomas (Hostetter et al., 2005). The lepromatous lesions consist primarily of macrophages containing large numbers of MAP and can be characterized by a Th2 cytokine response. Compared with a tuberculoid-type granuloma, there is greater expression of IL-4 and IL-10 in the lepromatous group. The end result to the host in the advanced stage of JD is a corrugated and thickened instestinal mucosa (Clark, 1997). The sloughing of the mucosa during this stage results in increased shedding of MAP in the feces. The terminal stage of disease also is characterized by severe diarrhea. Inflammation and thickening of the intestinal mucosa results in reduced protein absorption, and fluid is not sufficiently absorbed. This directly results in an increase of water in the feces.

Lymphocytes and Johne's Disease

There are two distinct lineages of T-cells based upon the T-cell receptor, αβ and γδ, with commitment to a lineage occurring early in T-cell development (MacDonald et al., 2001). T-cells committing to the αβ lineage will develop further into either a $CD4^+$ or $CD8^+$ phenotype. Both the duration and strength of the T-cell receptor signaling control the CD4-CD8 lineage. A longer duration of signal results in the propagation of $CD4^+$ T cells, and a repression of $CD8^+$ T cells is required for $CD4^+$ lineage commitment (Liu and Bosselut, 2004). Likewise, an increase in signal strength results in the $CD4^+$ lineage (Watanabe et al., 2000).

$CD4^+$ T Lymphocytes

$CD4^+$ T-cells, also known as T helper cells, play an active role in initiating both the humoral and cell-mediated immune responses. Naïve $CD4^+$ cells are uncommitted and may proliferate into a Th0 immature effector T-cell. The immature cell can differentiate into Th1, Th2, Th3, or Treg cells. Which lineage is chosen is based on the cytokine microenvironment surrounding the infectious agent. Cytokines, such as IFN-γ and IL-12, will drive Th0 cells to a cell-mediated, or Th1, immune response. Production of IL-12 from macrophages and dendritic cells (DC) acts on naïve $CD4^+$ T-cells to direct development towards a Th1 response (Macatonia et al., 1995). In contrast, the presence of IL-4 will drive a humoral, or Th2, immune response. The differentiation of the Th0 cell is critical in controlling the outcome of intracellular infection.

The percentage of $CD4^+$ T-cells in PBMCs from healthy adult dairy cattle is approximately 25-35%. In mycobacterial infections, $CD4^+$ T-cells are recognized as the primary producers of IFN-γ (Stuehr and Marletta, 1987; Flynn et al., 1993). The effects of MAP on the percentages of $CD4^+$ T-cells are unclear. One study did not observe differences in the percentage of $CD4^+$ T-cells in fresh PBMCs isolated from MAP-infected periparturient cows (Harp et al., 2004). However, another study found that the ileum and peripheral blood of clinically infected cows have fewer $CD4^+$ cells than subclinically infected cows (Koets et al., 2002).

$CD8^+$ T Lymphocytes

$CD8^+$ or cytotoxic T-cells are important for their ability to recognize viral and bacterial antigens and target dendritic cells displaying these antigens for apoptosis (Kagi et

al., 1994). These cells release granules that contain perforin and granzymes. $CD8^+$ cells also express a Fas ligand, which, upon binding, can activate apoptosis. This T cell subset recognizes the MHC class I molecule. MHC class I molecules present peptides generated in the cytosol to $CD8^+$ T-cells. The importance of a functional MHC class I was highlighted by Flynn et al. (1992) who developed a mouse with a disruption in the β2-microglobulin. These mice did not have a functional MHC class I. Infection with *M. tuberculosis* resulted in the death of 70% of the knockout mice after 6 weeks compared with 20 weeks for the controls. The knockouts also had a greater number of tubercle bacilli present in lung tissue. The importance of MHC I in controlling mycobacterial infections was highlighted further in a study that reported mice deficient in transporter associated with antigen processing (TAP) 1 were more susceptible to intravenous infection with *M. tuberculosis* (Behar et al., 1999). TAP1 is located in the endoplasmic reticulum (ER) and mediates the active transport of peptides from the cytosol into the lumen of the ER. These TAP1 KO mice had 10-100 fold increase in the number of bacteria isolated from their lungs and had a reduced survival rate. Studies using Fas and perforin knockout mice have shown that mice challenged with mycobacteria do not rely on Fas or perforin-mediated effects for the early control of infection (Cooper et al., 1997a; Laochumroonvorapong et al., 1997). The secretion of cytokines is likely very important for the contribution of $CD8^+$ cells to the host immune response against MAP.

Cytokine secretion by the $CD8^+$ cells contributes to the host immune response to invading mycobacteria. Similar to $CD4^+$ T-cells, $CD8^+$ cells also secrete the proinflammatory cytokines, IFN-γ and TNF-α, making them equally important in controlling infection (Fong and Mosmann, 1990; Berg et al., 2002). $CD8^+$ T-cells also have the capacity

to produce Th2 cytokines. Stimulation of rat spleen with PMA and ionomycin resulted in an upregulation of IL-4, IL-5, IL-10, and IFN-γ (Noble et al., 1995). $CD8^+$ cells also secrete TGF-β and IL-4 (Weiner et al., 1994).

Studies depleting mice of their $CD8^+$ T-cell populations by using anti-CD8 antibodies have demonstrated a protective role for $CD8^+$ cells in *M. tuberculosis* infection (Muller et al., 1987). Activation of $CD8^+$ in response to live *M.* bovis has been reported in human PBMCs (Turner et al., 1996). Studies in healthy dairy cows support a decline in $CD8^+$ cells at parturition (Kimura et al., 1999; VanKampen et al., 1999). This decline is in contrast to two studies reported by Harp et al. (1991 and 2004) that suggested $CD8^+$ T-cells in peripheral blood were not influenced by parturition. MAP infection does not seem to affect the percentage of $CD8^+$ cells (Koets et al., 2002).

γδ T-Lymphocytes

The γδ T-cell population is a distinctly different subset of T-cells that are not characterized by the αβ TCR chain. γδ T-cells seem to initiate immune responses and may regulate host inflammatory response to infection (King et al., 1999; Tagawa et al., 2004). However, their specific roles in host immunity are still relatively undefined. These cells are unique in that they do not utilize MHC complexes to recognize antigens. The percentage of γδ T-cells in blood is greatest in the calf (40%) and gradually declines to approximately 5% of adult PBMCs (Hein and MacKay, 1991). In ruminant animals, a subset of the γδ T-cell population expresses $WC1^+$ on their surface. Observations for periparturient dairy cows suggest that percentages of γδ T-cells either decline (VanKampen and Mallard, 1997; Kimura et al., 1999) or remain unchanged (Park et al., 1992; Harp et al., 2004; Meglia et al., 2005).

WC1.1+ cells are the primary source for IFN-γ derived from γδ T-cells (Rogers et al., 2005). In addition to IFN-γ, γδ T-cells also secrete IL-2 and TNF-α (Christmas et al., 1990).

A role exists for this subpopulation of T-cells in the early stages of intracellular infections. These cells play a regulatory role by influencing the movement and function of the inflammatory effectors, neutrophils (Fu et al., 1994) and macrophages (Tagawa et al., 2004), to the site of infection. This is an important point since the formation of granulomas at the site of infection is a hallmark of Johne's disease. Mice infected with live *M. bovis* BCG had a large accumulation of γδ T-cells at the site of infection (Griffin et al., 1991). Koets et al. (2002) reported that the population of γδ T-cells in freshly isolated PBMCs was much greater in control cows compared with MAP-infected clinical cows. The early stages of infection with *M. tuberculosis* resulted in increased numbers of activated γδ T-cells that were capable of producing IFN-γ (Tsukagucki et al., 1995). The secretion of IFN-γ by the γδ T-cells contributes to the early protection needed before the initiation of the αβ T-cell response. The exact role of γδ T-cells in MAP-infection remains unclear. However, studies suggest that γδ T-cells plays an initial role in the host immune response to the invading MAP bacteria.

B Lymphocytes

As Johne's disease progresses, there is a transition from the cell-mediated to humoral immune response. B-cells are responsible for the induction of the humoral response. Upon activation, B cells differentiate into antibody-secreting plasma cells. The presence of helper T cells and antigen are needed to support this activation that occurs by the binding of the antigen to the B-cell receptor. Secreted antibodies can neutralize or opsonize the pathogen or

activate complement. Isotype switching of the secreted antibody results in IgG, IgA, IgM, IgD, or IgE. Cytokines are capable of inducing isotype switching. IFN-γ is essential for the class-switching to IgG_{2a} and IgG_3 (Snapper and Paul, 1987), whereas, the Th2 cytokine IL-4 induces the expression of IgG_1 and IgE (Abbas et al., 1996). The antibodies IgG and IgM are expressed in high concentrations in serum of cattle naturally infected with MAP (Abbas and Riemann, 1988). In addition to serum, IgG and IgM also were found in greater quantity in the ileum of MAP-infected cows compared with noninfected animals (Momotani et al., 1986). Both of these classes of immunoglobulins promote neutralization of bacteria and activate the complement system.

In the advanced stages of JD, antibody production by B-cells does little to protect the host from the progressive MAP-infection. B-cells from clinical, but not subclinical JD cows, fail to proliferate in response to antigen stimulation (Waters et al., 1999). Furthermore, the percentages of B-cells in peripheral blood are similar between subclinical and control cows but greater in clinical cows (Waters et al., 1999). Coussens et al. (2002) reported a repression of the Lyn B protein tyrosine kinase in clinical cows compared with that in subclinical and control cows. The Lyn B gene is involved in B-cell signaling through the B-cell antigen receptor. The periparturient period does not have an effect on B-cell proliferation (Nagahata et al., 1992). However, decreased IgM secretion immediately before calving has been reported (Lacetera et al., 2005). Decreased MAP-specific serum antibody was observed after calving in dairy cows with JD (Stabel and Goff, 2004).

Cytokine Regulation of Johne's Disease

The development and progression of Johne's disease is influenced greatly by the cytokine microenvironment. Cytokines are small proteins or peptides released from cells that have a specific effect on the behavior of cells. Although $CD8^+$ and γδ T-cells secrete cytokines, the $CD4^+$ subset plays the primary role in controlling MAP infection through cytokine production. In 1989, Mosmann et al. (1986) described the formation of two distinct populations of mouse $CD4^+$ T-cells that could be distinguished on the basis of their patterns of cytokine production. These populations are called Th1 and Th2, and the interaction of the two results in a polarization of the cytokine response. Naïve $CD4^+$ T-cells have the potential to develop into a Th1, Th2, Th3, or Treg cell. This section of the literature review presents data that support a role for the Th1 cytokines in subclinically infected dairy cows. As paratuberculosis progresses to the clinical stage, there is a transition from a Th1 to a Th2 cytokine response. A summary of the Th1/Th2 paradigm is provided in figure 1.1.

T-helper 1, Proinflammatory Cytokines

An effective Th1 response to MAP infection is critical for controlling the initial stages of the disease. The secretion of Th1 cytokines is primarily responsible for the induction of a cell-mediated immune response. Classical Th1 cytokines include IL-1α, IL-1β, IL-8, IL-12, IL-18, IFN-γ, and TNF-α (Fig. 1.1). Of the classical cytokines, IFN-γ is among the first to be activated in subclinically infected cows (Sweeney et al., 1998).

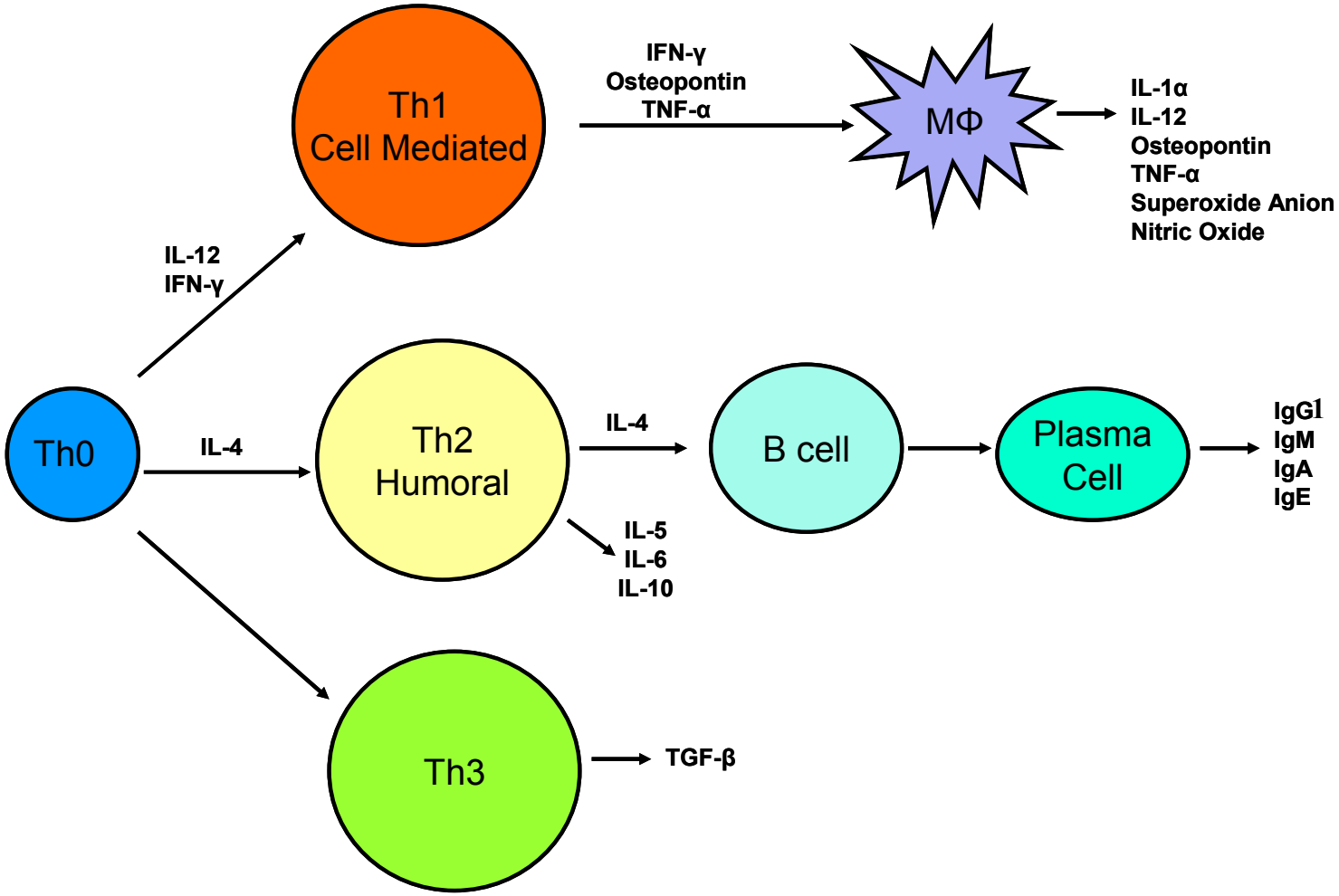

Figure 1.1 The Th1/Th2 Paradigm.

Interferon-γ is a Type II interferon and is secreted by $CD4^+$ Th1 cells, $CD8^+$ T-cells, γδ T-cells, and activated natural killer cells. Biologically active IFN-γ is a noncovalent linked homodimer (Ealick et al., 1991). Interferon-γ has two receptors that are members of the class II cytokine receptor family. The first chain, IFNGR1, is the binding chain, and the second chain, IFNGR2, is responsible for signal transduction (Aguet et al., 1988). However, the IFNGR1 chain is produced in surplus (Bernabei et al., 2001) and has binding motifs for Janus tyrosine kinase (Jak)1 and signal transducer and activator of transcription (Stat)1. Interferon-γ binds to IFNGR1, and then this complex associates with IFNGR2. Ligand binding phosphorylates Jak1, which, in turn, phosphorylates Stat1. The Stat1 homodimer translocates to the nucleus where it binds to promoter elements to influence gene expression. The importance of functional IFN-γ receptor recognition is evident when mice lacking the IFN-γ receptor are infected with the BCG strain of *M. bovis*. Infection is lethal for deficient mice but not for the wild-type mice with a functional receptor (Kamijo et al., 1993). Micewith disrupted IFN-γ gene do not survive when exposed to sublethal doses of *M. bovis* (Dalton et al., 1993).

Tumor necrosis factor-α was first described in 1985 by Lloyd Old. Tumor necrosis factor-α is predominantly involved in local and systemic inflammation and is a major mediator of endotoxic shock. It is produced by macrophages, T cells, mast cells, and natural killer cells. There are two receptors for TNF-α, TNF-R1 and TNF-R2. TNF-R1 is constitutively expressed by all mammalian cells, but TNF-R2 is only expressed by immune cells (Tartaglia et al., 1991). However, the majority of information on the TNF-α receptors is for the TNF-R1. Upon engagement of TNF-α and its receptors, a trimer is formed and

conformational changes in the receptor occur. These changes cause the disassociation of silencer of death domains (SODD) (Takada et al., 2003) and the subsequent binding of TNFRSF1A-associated via death domain (TRADD) to the death domain. After TRADD binds, there are three potential pathways that can be activated. These are the activation of NF-κB, activation of MAPK pathways, or the induction of death signaling via caspase 3. These three distinct pathways ultimately will result in cellular proliferation, inflammation, or apoptotic cell death.

Peritoneal mast cells constitutively contain large amount of TNF-α in their granules (Gordon and Galli, 1990). In addition to causing inflammation, TNF-α enhances the growth and maturation of B cells (Jelinek and Lipsky, 1987) and causes upregulation of MHC class I on endothelial cells. It is interesting to note that TNF-α induces its own mRNA in inflammatory peritoneal macrophages (Kindler et al., 1989). Tumor necrosis factor-α is involved in the early stages of MAP infection by controlling bacterial proliferation (Appelberg, 1994). This cytokine is of critical importance in the formation of granulomas in response to MAP infection. Treatment with anti-TNF-α antibodies prevents the development of granulomas. Particles that are left undigested within macrophages lead to an increased and sustained release of TNF-α (Stein and Gordon, 1991). Partial digestion is consistent with the behavior of phagocytized MAP. Isolated PBMCs from subclinical cows produced more TNF-α when stimulated with a whole-cell sonicate of MAP compared with PBMCs from control and clinical cows (Stabel, 2000). Stimulating PBMCs from MAP-infected castrated male Holstein calves with live MAP resulted in significantly higher TNF-α gene expression (Buza et al., 2003). These studies highlight the importance of TNF-α secretion in the immune response to MAP infection.

Interleukin-12 is secreted by activated macrophages, natural killer cells, and T cells. It plays an active role in inducing $CD4^+$ T cells to differentiate to Th1-like cells and secrete IFN-γ (Kaplan et al., 1996). Interleukin-12 has a heterodimeric structure that is composed of two subunits, p35 and p40, linked by a disulfide bond. Each subunit is unique in that it is expressed by its own gene and is on a different chromosome (Brombacher et al., 2003). However, coexpression of both subunits is required for the secretion of biologically active IL-12 (Gubler et al., 1991). Interleukin-12 receptors are primarily expressed on activated T cells and natural killer cells (Desai et al., 1992). The two type I transmembrane glycoprotein receptors, IL-12Rβ1 and IL-12Rβ2, form dimers, and their coexpression is required for high-affinity IL-12 binding (Presky et al., 1996). Interleukin-12 is able to induce tyrosine-phosphorylation of Stat4, which induces naïve T-cells towards a Th1 phenotype. The importance of Stat4 to the IL-12-mediated immune responses was highlighted in a study utilizing *stat4*$^{-/-}$ mice. Stimulating the spleen cells of Stat4 knock-out mice with IL-12 resulted in no detectable production of IFN-γ (Thierfelder et al., 1996). In the absence of Stat4, IL-12 induced proliferation of lymphocytes is halted and there is an increase in Th2 lymphocytes (Kaplan et al., 1996).

The major role of IL-12 is to regulate the balance of Th1 and Th2 cells. It does this by promoting the differentiation of naïve T cells to Th1 cells. IL-12 induces the secretion of IFN-γ from activated T cells and NK cells (Puddu et al., 1997). When mice are treated with IL-12, an enhancement of IgG_{2a} and suppression of IgG_1 production is observed (Buchanan et al., 1995). Both IL-12 and IFN-γ support the differentiation of $CD8^+$ T-cells into active cytotoxic cells (Abbas et al., 1996).

The ability of IL-12 to induce the expression and secretion of IFN-γ, makes this cytokine of interest in MAP-infection. Neutralizing IL-12 in female BALB/c mice intravenously infected with 10^6 CFU of *M. avium* resulted in increased expression of both IL-4 and IL-10 mRNA and reduced expression of IFN-γ compared with the controls (Castro et al., 1995). Incubating monocytes isolated from adult healthy cattle for 4 hours with live MAP (10 bacilli/monocyte) reduced the expression of IL-12 (Souza et al., 2006).

Overall, the Th1 cytokines, representing the proinflammatory cytokines, predominate in the subclinical stage of Johne's disease. These cytokines are responsible for not only activating the innate immune response but also in influencing the differentiation of naïve $CD4^+$ T-cells to a Th1 response.

T-helper 2, Anti-inflammatory Cytokines

The transition from the subclinical to clinical state coincides with the differentiation of naïve $CD4^+$ T-cells to a Th2 immune response. The production of Th2 cytokines supports a humoral immune response by stimulating the proliferation of B lymphocytes and inhibiting Th1 cytokines. Classical Th2 cytokines include IL-4, IL-5, and IL-10.

Interleukin-10 was originally described in 1989 as a cytokine synthesis inhibitory factor that was secreted from Th2 T-cells, but not Th1 cells (Fiorentino et al., 1989). Interleukin-10 is produced by T cells, macrophages, and B cells (O'Garra et al., 1990) and functions to suppress the Th1 response. The receptors for IL-10 are IL-10R1 and IL-10R2. Both receptors are similar in structure and are members of the class II cytokine receptor family (Kotenko, 2002). Together the receptors make up four transmembrane polypeptides.

Two of the chains are for the IL-10R1 receptor and are responsible for ligand binding. The two other chains are for the IL-10R2 and initiate signal transduction. Upon binding of IL-10 to its receptors, Jak1 and Tyk2 are activated (Finbloom and Winestock, 1995). Jak1 is constitutively bound to IL-10R1. Macrophages from Jak1 knockout mice do not respond to IL-10 (Rodig et al., 1998). The activation of Jak1 and Tyk2 results in the phosphorylation of Stat3, Stat1, and Stat5. The Stat proteins form homodimers and heterodimers (Wehinger et al., 1996), translocate to the nucleus, and activate Stat3 responsive genes. Two of these activated genes are suppressor of cytokine signaling (SOC) 1 and 2. The activation of SOCS1 inhibits IFN-γ production by T-cells (Ding et al., 2003).

In human monocytes, IL-10 inhibits the production of the Th1 cytokines, IL-1α, IL-1β, IL-6, IL-8, TNF-α, GM-CSF, and G-CSF (de Waal Malefyt et al., 1991). Interleukin-10 functions to inhibit the expression of IFN-γ. Neutralizing IL-10 in MAP-infected PBMCs isolated from 40-month-old castrated male Holstein cattle resulted in a 9-fold increase in IFN-γ production (Buza et al., 2004). Interleukin-10 suppresses macrophage activation and downregulates the expression of the costimulatory molecules, CD80 and CD86, and MHC II (Willems et al., 1994).

Data suggest that IL-10 and TGF-β are upregulated in clinical MAP-infected dairy cows (Khalifeh and Stabel, 2004a). Treatment of PBMCs with exogenous IL-10 and TGF-β resulted in the downregulation of IFN-γ production (Khalifeh and Stabel, 2004a). PBMCs isolated from dairy cows naturally infected with MAP, had increased GATA 3 expression compared with control cows (Coussens et al., 2005). The transcription factor GATA 3 polarizes cells to a Th2-like phenotype. Infection of monocyte-derived bovine monocytes with MAP resulted in upregulation of IL-10 (Weiss et al., 2005). Furthermore, neutralizing

IL-10 caused a 57% increase in the ability of macrophages to kill MAP within 96 hours, an increase in the acidification of phagosomes, and an increase in the expression of IL-12 and TNF-α (Weiss et al., 2005). These experiments provide support for the anti-inflammatory, suppressive role of IL-10 in the progression of JD.

Interleukin 4 is produced by T cells, mast cells, and natural killer cells. The primary mode of initiating a response is by the engagement of IL-4 with its Type 1 cytokine receptor. The IL-4 receptor is composed of a common gamma chain (γc) and an alpha chain (α) (Nelms et al., 1999). The IL-4Rα chain has a high affinity for IL-4 (Galizzi et al., 1990) and IL-4 will bind with this chain first. It is also a component of the IL-13 receptor (Obiri et al., 1997). Upon binding, the complex dimerizes with IL-4Rγ chain (Mueller et al., 2002). The initial signaling steps occur with the phosphorylation of Jaks (Hershey, 2003). Additional phosphorylation of the tyrosine residues in IL-4Rα is required for the activation of Stat6 (Ryan et al., 1996). The complex then is translocated into the nucleus where it can affect gene expression. Interleukin-4 induced T cell differentiation occurs via Stat6 (Zhu et al., 2001). Mice deficient in Stat6 have impaired macrophage development and function, a decline in MHC class II expression, and a decline in nitric oxide production (Takeda et al., 1996).

The main function of IL-4 is to promote B-cell activation and to suppress the Th1 response. Interleukin-4 promotes the isotype switching of murine plasma B-cells to IgE (Abbas et al., 1996; Del Prete et al., 1988). In Stat6 knock-out mice challenged with *Nippostrongylus brasiliensis*, there was a 16-fold lower production of IgG_1 compared with the wild-type mice and undetectable production of IgE. In addition to the effect on isotype switching, there was also a decrease in the production of Th2 cytokines and B-cell

proliferation (Shimoda et al., 1996; Takeda et al., 1996). Secretion of IL-4 upregulates MHC class II production. Coussens et al. (2004) reported decreased IL-4 expression from PBMCs isolated from subclinical JD cows compared with clinical and control cows. Both IL-4 and IL-10 are expressed significantly more in the ileal lymph nodes of lepromatous lesions compared with tuberculoid lesions in dairy cows infected with MAP (Tanaka et al., 2005). Similarly, peripheral blood mononuclear cells isolated from patients with advanced disease caused by *M. tuberculosis* had increased IL-4 production compared with patients with mild TB. The IFN-γ/IL-4 ratio of the patients with mild TB were significantly higher than the patients with advanced TB (Dlugovitzky et al., 1999). Increased expression of IL-4 during the later stages of mycobacterial infections may have implications for the role of IL-4 in disease progression. Additional research is required to further characterize this potential role in MAP-infected dairy cows.

T-helper 3 Cytokines

A third type of naïve $CD4^+$ T-cell differentiation results in Th3 cells. These cells are responsible for the induction of TGF-β. In the mammalian species, there are three isoforms of TGF-β: TGF-β1, TGF-β2, and TGF-β3. However, isoforms 2 and 3 play insignificant roles in the immune system (Govinden and Bhoola, 2003). Mature TGF-β is a 24-kDa homodimer and is associated with a latency-associated peptide (LAP). This association is essential to insure proper secretion and correct folding of TGF-β. Once disassociation of TGF-β and LAP has occurred, TGF-β is free to bind to its receptor through two transmembrane serine/threonine receptor kinases (Rubtsov and Rudensky, 2007). It initially

binds to the type II receptor, which then recruits and phosphorylates the type I receptor. Receptor regulated Smad is subsequently phosphorylated and forms a heteromeric complex with Smad4 (Govinden and Bhoola, 2003). This complex translocates into the nucleus and is able to influence gene expression.

The immune functions of TGF-β are diverse. In 1986, it was first reported that TGF-β is produced by activated T-cells and has an autocrine function to inhibit the proliferation of these T-cells (Kehrl et al., 1986). Peripheral blood monocytes also have been shown to secrete TGF-β. Deletion of the TGF-β receptor II in mice, resulted in development of a lethal inflammation related to T-cell activation (Li et al., 2006). TGF-β and Th2 cytokines work synergistically to inhibit the secretion of IFN-γ and the activation of macrophages. Secretion of TGF-β1 is required to sustain the $CD4^+$ regulatory T-cell subset that expresses the transcription factor forkhead box P3 (Chen et al., 2003; Marie et al., 2005). T regulatory-1 cells are important for the production of IL-10 (Maynard et al., 2007). When human PBMCs are treated with PPD and recombinant TGF-β, there was a 70-fold increase in the production of IL-10 from monocytes compared with PPD treatment alone (Othieno et al., 1999). The effects of TGF-β on monocytes/macrophages include increasing chemotaxis but decreasing phagocytosis and antigen presentation (Rubtsov and Rudensky, 2007). TGF-β affects B cells by causing an increase in IgA class switching and by decreasing the activation, proliferation, and survival of the cells (Ohtsuka and Sanderson, 2000; Rubtsov and Rudensky, 2007).

Understanding the role of TGF-β in mycobacterial infections is important based on its interaction with a diverse array of immune cells. TGF-β is upregulated in clinical Johne's

cows (Khalifeh and Stabel, 2004a) and was found to be expressed 3.4-fold higher in PBMCs from infected cows compared with control (Coussen et al., 2005). When PBMCs were isolated from naturally infected cows and incubated with MAP, there was an increase in TGF-β expression in infected macrophages compared with nonactivated cells (Weiss et al., 2004). Importantly, the Th2 and Th3 cyokines have been shown to work synergistically. TGF-β and IL-10 act in concert to repress macrophage activation (Mullins et al., 2001). Interleukin-10 interacts with IL-4 and TGF-β to inhibit the production of nitric oxide from IFN-γ-activated macrophages (Oswald et al., 1992). The ability of MAP to modulate the Th2 and Th3 cytokines is critical in the pathogenesis of JD.

Cytokines and Mycobacterial Infections

During mycobacterial infection, the host is challenged with the initial need to control the invading organism. $CD4^+$ T-cells, of the Th1 phenotype, play a protective role in controlling mycobacterial infections. As previously stated, the acute phase of the disease is characterized by a Th1 response which gives way to a Th2 response during the chronic phase. Wangoo et al. (20001), in an effort to characterize the contribution of Th1 and Th2 cells to mycobacterial infection, used a murine model where splenocytes had a Th0 profile. The profile could be altered by *in vitro* culture conditions to polarize toward a Th1 or Th2 phenotype. Murine cells cultured with 10 U/mL IL-12 and 10 µg/mL anti-IL-4 Ab-generated cells with a Th1 profile and had a 70-fold increase in IFN-γ production. Cells cultured with IL-4 expressed a 20-fold increase in IL-4 expression. Furthermore, the bacterial load in the lungs of mice infected with *M. tuberculosis* in the presence of Th2 cells was 10-fold higher than in the presence of Th1 cells.

The importance of the Th1 response is highlighted in studies utilizing Th1 cytokine knock-out mice. When IFN-γ knock-out mice were infected intravenously with 10^5 *M. tuberculosis,* the mice lost their ability to control the bacteria (Cooper et al., 1993). The KO mice developed the fatal disseminated form of tuberculosis, and bacterial infection was present in the kidney and bone marrow. IL-12p40$^{-/-}$ knock out mice are unable to control bacterial growth when infected with 10^5 *M. tuberculosis*. All of the IL-12 KO mice had decreased secretion of IFN-γ and succumbed to the disease between 40 and 45 days post-infection (Cooper et al., 1997b).

It is well documented in *M. tuberculosis*-infected mice that the initial phase of infection is highlighted by a Th1 response. $CD4^+$ T-cells expressing a Th2 phenotype predominate during the chronic phase of the disease. When healthy mice were injected with 10^5 *M. tuberculosis,* the host acquired response was characterized by an early Th1 response (Orme et al., 1993). Secretion of IFN-γ was high for the first 30 days of infection, but then declined. As IFN-γ declined, there was an increase in production of the Th2 cytokine, IL-4 (Orme et al., 1993). A similar study examined the transition from a Th1 to Th2 response in mycobacterial infections by injecting mice with 10^6 *M. tuberculosis*. The authors reported that, as the disease progressed, there was a transition from an acute to chronic phase which was characterized by a decline in IFN-γ expression and an increase in IL-4 (Hernandez-Pando et al., 1996).

The magnitude of the Th2 response has been correlated with the extent of disease progression. In human patients infected with *M. tuberculosis*, expression of IL-4 mRNA was correlated with clinical measures of disease severity, with advanced disease resulting in increased IL-4 expression (Seah et al., 2000). The authors of this study concluded that Th2

cytokines are related to unfavorable disease outcomes. There is also a positive correlation between Th2 cytokine response and tissue necrosis in patients with pulmonary tuberculosis. As the disease progresses, there is a decline in the IFN- γ/IL-4 ratio (van Creval et al., 2000).

These studies provide evidence in support of a transition from a Th1 to a Th2 phenotype that can be correlated with progression of mycobacterial disease.

The Periparturient Period

Negative Energy Balance

The periparturient period, defined as three weeks prepartum through three weeks postpartum, represents a time of physiological stress for the dairy cow (Grummer, 1995). Although it is anecdotal information, field observations suggest that dairy cows transition from the subclinical to clinical stage of MAP infection during this stressful time. During the first week of lactation, the dairy cow does not maintain sufficient intake to support nutrient requirements for milk production (Bell, 1995). The recommended energy density in diets fed to periparturient dairy cows is 1.62 Mcal NE_L/kg DM (NRC, 2001). In the immediate post-partum time period, the dairy cow experiences a decline in dry matter intake (DMI), which coincides with an increased energy need to support the onset of lactation. Although a 30% reduction in DMI is typical during the last four weeks of gestation (Grummer, 1995), a decline up to 47% has been reported (Douglas et al., 2006). Reduction in DMI also occurs within the first few days after calving and may be depressed as low as 40% during the immediate postpartum period (Vazquez-Anon et al., 1994). Negative energy balance,

independent of parturition, may occur in subclinical MAP-infected dairy cows (Johnson-Ifearulundu et al., 2000). Although this suggestion is reasonable based on the reduction in intestinal function reported in subclinical cows further investigation is required.

The end result of the negative energy balance is the mobilization of body fat stores as non-esterified fatty acids (NEFAs). Serum NEFA concentrations are often elevated above 1000 μeq/L during the initial postpartum period (Karcher et al., 2007; Vazquez-Anon et al., 1994). One study examined the effects of feeding periparturient dairy cows naturally infected with MAP a diet at 2% of their body weight and then manually stuffing the orts into the rumen. Results showed that stuffed cows had a reduction in both serum NEFA and β-hydroxybutyrate concentrations (Stabel et al., 2003), suggesting that additional energy reduced the need to breakdown fat. Serum NEFAs also have been reported to play a role in the immune regulation by inhibiting the secretion of both IgM and IFN-γ at concentrations ranging from 0.25 – 2.0 mmol/L (Lacetera et al., 2004).

Nutritional and Immunological Challenges

In addition to the nutritional challenges the periparturient dairy cow faces there are also challenges to the immune system. Increases in both metabolic and infectious diseases are observed during this critical time period (Goff and Horst, 1997). The major incidences of metabolic disease occur during the first two weeks of lactation (Goff and Horst, 1997). Examples of metabolic diseases afflicting periparturient dairy cows include fatty liver, mastitis, hypocalcemia, and displaced abomasum. Both the innate and adaptive immune responses are lowest during the periparturient period (Mallard et al., 1998). Neutrophil function is compromised at parturition (Kehrli et al., 1989a), and the killing capacity of

neutrophils is diminished (Cai et al., 1994; Detilleux et al., 1995). Lymphocyte function is also diminished at parturition (Kehrli et al., 1989b). Studies with dairy cows have supported an increase (Van Kampen et al., 1999), decrease (Kimura et al., 1999), or no change (Harp et al., 2004) in the percentage of $CD4^+$ T-cells at parturition. Similar studies with healthy cows have reported a decline in $CD8^+$ T-cell percentages at parturition (Kimura et al., 1999; VanKampen et al., 1999). This change is in contrast to two studies reported by Harp et al. (1991 and 2004) that suggest that $CD8^+$ T-cells in peripheral blood are not influenced by parturition. Typical observations for dairy cows during the periparturient period include either declining numbers of γδ T-cells (Van Kampen and Mallard, 1997; Kimura et al., 1999) or no change in the percentage of γδ T-cells (Park et al., 1992; Harp et al., 2004; Meglia et al., 2005). Kimura et al. (1999) reported a decline in γδ T-cells from 7.3% on day -13 to 5.4% on day +5 relative to calving. There are also conflicting reports in the literature regarding B-cells. Some studies indicate that the percentage of B-cells in peripheral blood throughout the periparturient period remains constant at 25% (Park et al., 1992; Harp et al., 2004) whereas others indicate increasing (Meglia et al., 2005) and decreasing (Van Kampen and Mallard, 1997) percentages during the postpartum period. Detilleux et al. (1995) reported a depression of IgG_1, but an increase in IgG_2 at calving.

The transition to lactation is a complicated time for even healthy dairy cows. Further understanding of the immune response at this time is required and how immunosuppression during this time may result in the progression of JD.

Cytokine Expression and the Periparturient Period

In order to appreciate the effects of parturition on the progression of JD, it is important to understand the cytokine expression profile in healthy periparturient cows. Throughout mid to late lactation, cytokine profiles mirror a Th1 immune response. The expression (Shafer-Weaver et al., 1999) and secretion (Ishikawa et al., 1994) of IFN-γ begins to decline as parturition approaches. Contrary to IFN-γ, isolated mononuclear cells from periparturient dairy cows produce greater amounts of TNF-α than do mid to late lactating dairy cows (Sordillo et al., 1995). This finding contradicts the Th1/Th2 paradigm typically expressed by dairy cows at parturition. As the transition to lactation occurs, there is a shift in the $CD4^+$ T-cell population supporting a Th2 response (Shafer-Weaver et al., 1999). In healthy periparturient dairy cows, IL-10 expression is greater in cell cultures isolated immediately postpartum compared with mid to late lacatation (Shafer-Weaver et al., 1999). IL-4 is upregulated during the immediate postpartum period, and production is lowest in mid to late lactation cows (Shafer-Weaver and Sordillo, 1997; Shafer-Weaver et al., 1999). The Th2 response in humans has also been characterized during pregnancy. Increased expression of IL-10 and IL-10R1 has been observed in humans in the early stages of pregnancy (Vigano et al., 2002). Taken together, these studies suggest the immediate postpartum period favors a Th2 response in cattle.

Periparturient Hormonal Regulation of Cytokines

Hormonal fluctuations during the periparturient period contribute to the typical immunosuppression observed at parturition. The polarization of a specific Th response may be influenced by the hormonal status of the animals. Progesterone and estradiol are both key

pregnancy hormones that have been shown to affect the differentiation of $CD4^+$ T-cells. In periparturient dairy cows, progesterone concentrations decline at parturition and remain low during the initial weeks of lactation (Radcliffe et al., 2003). High circulating concentrations of progesterone and 17β-estradiol near the onset of parturition can suppress lymphocyte function (Medina and Kincade, 1994; McMurray et al., 2001). Progesterone is capable of up-regulating the Th2 response and contributes to the natural suppression of cell-mediated immunity. When naïve murine T cells were exposed to progesterone, there was an induction of IL-10- and IL-4-producing Th2 cells (Miyaura and Iwata, 2002). Interleukin-5 mRNA expression also was increased in progesterone-treated T-cells (Wang et al., 1993). Progesterone inhibited nitric oxide production and decreased inducible nitric oxide synthase (iNOS) gene promoter activity in IFN-γ (10 U/mL)- stimulated murine macrophages (Miller et al., 1996). In contrast, estradiol enhances Th1 development (Maret et al., 2003). *In vitro* estrogen treatment enhanced the anti-*Mycobacterium avium* complex (MAC) killing activity of macrophages isolated from mice injected intratracheally with MAC (50 µl bacterial suspension containing 2 x 10^5 CFU) (Tsuyugucki et al., 2001). Stimulating human PBMCs with 0.5 – 30 ng/mL of 17β-estradiol enhanced pokeweed mitogen-induced generation of antibody-secreting cells (Sthoeger et al., 1988). Furthermore, estrogen enhances phagocytosis by macrophages isolated from gonadectomized Balb/c mice (Baranao et al., 1991).

In periparturient dairy cows, insulin-like growth factor (IGF)-1 concentration in the blood and liver begins to gradually decline at day -14 relative to calving and then rapidly declined at parturition (Kobayashi et al., 1999). The expression of IGF-1 decreases in tissues when fasting occurs. Macrophages and B-cells secrete IGF-1 (Merimee et al., 1989), and

physiological concentrations of IGF-1 are capable of increasing IFN-γ (Arkins et al., 1993) and TNF-α (Renier et al., 1996) production by macrophages. IGF-1 skews cytokine production to the Th1 response and supports a proinflammatory role.

Finally, the steroid hormone cortisol is a member of the immunosuppressive glucocorticoid family. Cortisol is secreted from the adrenal cortex. Blood serum concentrations of cortisol dramatically increase at parturition and then decline during the postpartum period (Mallard et al., 1997). Cortisol influences the differentiation of naïve $CD4^+$ T-cells by promoting the secretion of Th2 cytokines. Glucocorticoid analog dexamethasone (DEX)-stimulated rat $CD4^+$ T-cells to express more IL-4, IL-10, and IL-13 and less IFN-γ and TNF-α compared with nonstimulated controls (Ramirez et al., 1996). Interleukin-12 production is suppressed by DEX treatment (Visser et al., 1998). Parturition affects the expression of the glucocorticoid receptor (GR) in neutrophils. Preisler et al. (2000) noted a 49% reduction in GR expression at calving compared with expression 2 to 4 weeks before calving. In addition to expression of GR, Fas expression decreases between calving and 12 hours postpartum (Chang et al., 2004) and downregulates apoptosis in circulating cells. When *M.tuberculosis*-infected human PBMCs were stimulated with physiological concentrations of cortisol, there was a decline in the mycobacterial antigen-driven proliferation of the cells (Mahuad et al., 2004). Furthermore, it seems that the growth of *M. tuberculosis* is not inhibited by the presence of glucocorticoids and that glucocorticoids can overcome the inhibition of growth observed by stimulation with recombinant IFN-γ (Rook et al., 1987).

Osteopontin

Osteopontin (Opn), also identified as early T cell activator-1 (Eta-1), is a highly acidic glycoprotein that is produced by both immune and non-immune cells. It was first isolated in 1985 from the mineralized matrix of bovine bone (Franzen & Heinegard, 1985). Non-immune sources of Opn include osteoclasts, osteoblasts, smooth muscle cells, epithelial cells, and endothelial cells. Osteopontin also can be isolated from physiological fluids--urine, milk, and seminal plasma (Cancel et al., 1997)-- and can be detected in the human glandular epithelial cells that line the stomach, small intestine, appendix, and bowels (Brown et al., 1992). Isolation of Opn from bovine milk revealed concentrations of 8 mg/L (Bayless et al., 1997). This is significantly higher than the 3 to 10 μg/mL reported in human milk (Senger et al., 1989). In healthy gut tissue, Opn is expressed by a subset of lamina propria mononuclear cells (Gassler et al., 2002). Osteopontin is most abundant in bovine bone cells and is barely detectable in bovine liver tissue (Kerr et al., 1991). Among its many functions, the ability of Opn to modulate the immune system to a Th1 response has earned it recognition as an important cytokine in controlling intracellular infection.

Structure and Function

Bovine Opn is a 278 amino acid protein and has a predicted molecular mass of 40 kDa (Kerr et al., 1991). The molecular mass of Opn from bovine milk is 66 kDa (Sorensen and Petersen, 1994). All mammalian species express Opn, and the cDNA and deduced protein sequences have been determined for the rat, mouse, pig, and human. Bovine Opn is most closely related to the pig with 71% identity and 83% similarity (Kerr et al., 1991). The

reported molecular mass of the protein will vary depending on the species and the tissue source. Evaluation of murine Opn revealed a molecular mass of 32 kDa and 297 amino acids (Young et al., 1990). Despite conservation among mammalian species, bovine Opn differs from others by a 22 amino acid deletion (Kerr et al., 1991). Osteopontin from all mammalian species has an arginine-glycine-aspartate (RGD) component at bp 517-525. The RGD site functions in cell attachment and exerts its effects by interacting with the $\alpha_v\beta_3$ integrin on macrophages. There are also hydroxyapatite (Oldberg et al., 1986) and calcium-binding domains (Patarca et al., 1993). Osteopontin lacks a membrane-anchoring domain and contains two heparin-binding domains (Patarca et al., 1993).

Osteopontin is secreted in both phosphorylated and nonphosphorylated forms. The various functions of Opn are made possible by extensive posttranslational modifications. These are primarily phosphorylation and glycosylation. Bovine Opn has 60 potential phosphorylation sites (Kerr et al., 1991), and it is phosphorylation that regulates the biological activity of the secreted cytokine. Evaluation of bovine milk Opn revealed 27 phosphoserines and one phosphothreonine (Sorensen et al., 1995). The most recognized biological activity of Opn lies within the N-terminal thrombin cleaved fragment. Thrombin cleavage regulates cell-receptor interactions with the RGD motif of Opn (O'Regan et al., 1999).

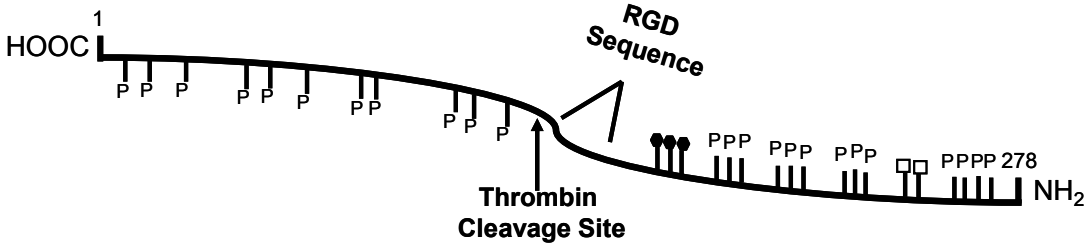

Figure 1.2. Bovine osteopontin protein with potential posttranslational modifications. Adapted from Sorensen et al., 1995. P- phosphorylation sites at serine; □ – transglutaminase reactive glutamines; hexagon – threonines.

As previously stated, the molecular mass of the secreted Opn varies depending upon the tissue and mammalian species. In general, Opn is between 44 and 75 kDa. Differences in molecular mass can be explained by extensive phosphorylation and N-linked glycosylation (Singh et al., 1990). The posttranslational modifications of Opn are dependent on the tissue in which it resides, and it is these modifications that allow for the multiple functions of the protein. Osteopontin has been identified in the Holstein bull reproductive tract and seminal plasma as three proteins at 55, 45, and 14 kDa (Cancel et al., 1997; Cancel et al., 1999). To date, there is a very small pool of literature evaluating Opn expression in bovine tissues and immune cells.

Innate Immune Response

Osteopontin plays an early role in initiating the innate immune response by promoting cellular adhesion and recruitment, increasing the activation of macrophages, and eliciting a Th1 cytokine response. Monocytes do not secrete Opn, but Opn is upregulated when monocytes differentiate into activated macrophages (Atkins et al., 1998). In healthy normal

tissue, the majority of Opn is expressed in bone and epithelial tissue (Brown et al., 1992). Although Opn is expressed constitutively, it is upregulated in inflamed tissues such as granulomas (Nau et al., 1997).

The importance of macrophages in the innate immune response has been discussed in previous sections. Osteopontin and macrophages interact through two receptors: $\alpha_v\beta_3$ integrin and CD44. The CD44 receptor mediates chemotactic migration. In 1990, it was reported that Opn binds to murine macrophages through a RGD-containing domain (Sing et al., 1990). Several years later, it was discovered that there was a receptor-ligand interaction between CD44 and Opn (Weber et al., 1996). Research by the same group later showed that the C terminal domain of Opn interacted with CD44 and that this engagement induced chemotaxis and chemoattractant activity (Weber et al, 2002). The N-terminal domain contains the $\alpha_v\beta_3$ integrin, which induces haptotaxis, adhesion, and spreading (Weber et al., 2002). When rats were injected intradermally with 300 ng of recombinant Opn, there was a 225% increase in the number of macrophages present at the injection site compared with the control (Giachelli et al., 1998). The Opn promoter region contains a vitamin D_3 response element and when macrophages were stimulated with vitamin D, upregulation of Opn transcription and protein secretion occured (Denhardt and Guo, 1993).

Stimulation of murine macrophages with osteosarcoma-derived Opn caused an increase in the production of the inflammatory cytokines, IL-12 and TNF-α (Weber et al., 2002), but not IL-10 or IL-6. This phenomenon is a result of the engagement of the $\alpha_v\beta_3$ NH_2-terminal fragment (Ashkar et al., 2000) and occurs in a phosphorylation-dependent manner. Stimulation of human PBMCs with 1 μg/mL of Opn resulted in a 5-fold increase in the production of IL-12 compared with stimulation of anti-CD3 alone (O'Regan et al., 2000).

Production of IL-12 as a result of Opn stimulation results in activation of additional macrophages and subsequent secretion of Opn. The presence of IFN-γ is required for the Opn-regulated production of IL-12 (O'Regan et al., 2000). There was a 15-fold increase in Opn promoter activity when a human monocyte/macrophage cell line was treated with exogenous IFN-γ (Li et al., 2003). These studies highlighting the importance of IFN-γ in Opn stimulation are further examples of an elegant feedback loop. Osteopontin knock-out mice have a 95% and 90% decrease in IL-12 and IFN-γ secretion, respectively, compared with the wild-type controls (Ashkar et al., 2000). In addition to IFN-γ, stimulating macrophages with TNF-α resulted in an upregulation of Opn mRNA and protein (Denhardt and Gou, 1993).

The ability to inhibit Th2 cytokines further establishes the role of Opn as a Th1 cytokine. Inhibition of IL-10 expression by Opn occurs by the phosphorylation-independent engagement of CD44 (Ashkar et al., 2000). Interestingly, Opn-deficient mice have increased IL-10 production and decreased macrophage function (Ashkar et al., 2000). Osteopontin stimulation also increases macrophage infiltration. Osteopontin inhibits gene transcription and protein expression of iNOS by the degradation of STAT1 in murine macrophages (Gao et al., 2007). This directly downregulates nitric oxide synthesis by murine macrophages (Guo et al., 2001)

In addition to macrophages, DCs play a major role in the innate immune response. Treatment of human DCs with 0.5 μg/mL Opn, induces DC differentiation, the upregulation of HLA-DR, CD40, CD80, CD86, CD44, and CD54 and promotes the emigration of Langerhans cells from the epidermis (Renkl et al., 2005). Furthermore, incubating human derived-DCs with 0.5 μg/mL of Opn stimulated secretion of TNF-α and IL-12; however, IL-

10 secretion was not affected (Renkl et al., 2005). Immature DCs secrete Opn, which, in turn, enhances the differentiation and maturation of DCs (Kawamura et al., 2005). This mechanism is similar to the one seen with the regulation of Opn and macrophages. In fact, murine Langerhans cells migrate to sites of high Opn expression in peripheral tissues (Weiss et al., 2001). Dendritic cells interact with Opn through the CD44 and $\alpha_v\beta_3$ receptors (Weiss et al., 2001). The ability of Opn to stimulate the activation of DCs and alter the cytokine microenvironment is key to a successful innate immune response.

Neutrophils are also key players in the innate immune response. However, compared with macrophages, neutrophils express much lower levels of Opn cDNA and Opn does not seem to be associated with the CD44 receptor (Koh et al., 2007). Deletion of Opn in the $Opn^{-/-}$ murine model reduced the recruitment of neutrophils by 2-fold (Koh et al., 2007). Experiments evaluating the relationship between Opn and neutrophil recruitment in women suffering from alcoholic liver disease revealed that Opn upregulated the CD11b neutrophil adhesion molecule (Banerjee et al., 2006). Despite its apparent role in increasing chemotaxis of neutrophils, it does not seem that neutrophil function was affected by the absence of the cytokine. Neutrophils isolated from osteopontin-null mice did not have any impairment of phagocytosis, cytokine production, or generation of reactive oxygen species (Koh et al., 2007).

Overall, Opn is a critical Th1 cytokine that plays a fundamental role in activating and recruiting the various components of the innate immune system.

Adaptive Immune Response

Osteopontin plays an integral role in the cell-mediated immune response. It is well established that activated T cells secrete Opn (Singh et al., 1990; Ashkar et al., 2000). Murine macrophages express approximately 10^4 Opn receptors/cell (Singh et al., 1990). Osteopontin induces T cell chemotaxis and costimulates T cell proliferation (O'Regan et al. 1999). When human T cells were stimulated for 24 h with anti-CD3 mAb and 1 μg/mL of Opn, there was an IL-12-dependent 4-fold increase in the production of IFN-γ than with anti-CD3 mAb alone (O'Regan et al., 2000). Upregulation of IFN-γ by activated $CD4^+$ T-cells is important because IFN-γ is needed to further activate macrophages. This same study evaluated the effects of Opn stimulation on CD40 ligand expression and confirmed that Opn costimulation of CD3-positive T cells upregulated CD40L expression (O'Regan et al., 2000). The upregulation of CD40L is crucial in the adaptive immune response because it increases the potential for the interaction with an antigen presenting cell. There was a dose-dependent increase in the production of IFN-γ, TNF-α, and IL-10 when human gut-derived T-cells were stimulated with milk-derived purified bovine Opn (0.01 – 10.0 μg/mL) and CD3 (0.1-0.5 μg/mL) antibody (Agnholt et al., 2007). Osteopontin also may promote the survival of activated T cells. When 5 μg of carrier-free mouse recombinant Opn was added to cultures of stimulated murine T-cells, there was an increase in the transcription factor NF-κB (Hur et al., 2007). Inhibition of forkhead box 03A and the proapoptotic proteins Bim, Bak, and Bax also was observed (Hur et al., 2007).

In addition to its effects on T-cells, Opn has an effect on B cells. Using a murine model, 5 μg/mL of recombinant Opn stimulated IgM and IgG production from a B cell/macrophage mixture (Lampe et al., 1991). It is not known if Opn interacts directly with

the B cell or if this is an indirect effect through macrophage cytokine production. Sato et al. (2005) showed that the primary producers of Opn in human inflamed intestinal tissue were primarily IgG^+ plasma cells. The majority of these Opn-producing IgG^+ plasma cells were IgG_1^+ and IgG_2^+.

Osteopontin and Intracellular Bacterial Infections

Based on its ability to upregulate and promote pro-inflammatory cytokines, the role of Opn in the host immune response to intracellular bacterial infections has been studied. To date, there are no studies evaluating the role of Opn in infections caused by MAP. However, there are an extensive number of human and murine studies documenting interactions between mycobacterial infection and Opn.

As previously discussed, during the subclinical stages of JD, there is formation of granulomas. Osteopontin is associated with $CD3^+$ T-cells in sarcoid granulomas, and Opn plasma concentrations are elevated in human patients with sarcoidosis compared with healthy controls (O'Regan et al., 1999). Staining of tuberculous lung sections from human patients infected with *M. tuberculosis* with anti-Opn detected Opn in the inflammatory border surrounding necrotic tissue and an overall increase in expression compared with normal lung sections (Nau et al., 1997). Osteopontin promotes chemotaxis and cellular adhesion of murine monocytes and macrophages (Weber et al., 2002). These effects, combined with its ability to anchor cells, link Opn to the initial stages of granuloma formation.

Crohn's disease (CD) is characterized by severe inflammation of ileal tissue and formation of granulomas. Plasma Opn is increased significantly in patients with active CD (Agnholt et al., 2007; Sato et al., 2005). Patients with active CD also exhibit upregulation of

Opn in the intestinal mucosa and immunohistochemical analysis of the mucosa detected Opn in areas surrounding granulomas (Sato et al., 2005). There is an inverse correlation between the concentration of Opn in tissues and disease severity in patients with mycobacterial infection (Nau et al., 2000). Patients suffering from nontuberculous mycobacterial infections with high Opn expression in infected lymph nodes recovered faster from the disease than did those patients with decreased Opn concentrations and disseminated infection (Nau et al., 2000). The relationship between inflammatory disease and Opn is important because of the ability of Opn to influence cytokine production towards a Th1 immune response.

Infections caused by *M. tuberculosis* are of particular concern because of its impact on world economics and health. *M. tuberculosis* infections are characterized by granuloma formation. A sufficient Th1 response it thought to be needed in the development of protective immunity against intracellular infections. Upregulation of Opn during infection offers the host protection during the initial stages. In fact, when human alveolar macrophages were infected with *M. tuberculosis* (bacteria:macrophage ratio of 10:1), Opn gene expression increased (Nau et al., 1997). To better understand the role of Opn in mycobacterial infections, Nau et al. (1999) utilized a murine Opn-KO mouse model. The mutant mice were challenged intraperitoneally with 10^7 CFU of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG). Compared with the controls, the mutant mice had more severe infection, heavier bacterial loads, and greater granuloma burdens. The macrophages from these mice were defective in killing BCG as evidenced by the delayed clearance of the bacteria. The results of this study support the hypothesis that Opn is a key player in the host response to intracellular bacteria. When granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced human macrophages were cultured with BCG (10 mg/mL) for three

hours, there was an upregulation of Opn (Khajoee et al., 2005). Furthermore, stimulation of the GM-CSF-induced human macrophages with Opn (2.5 μg/mL) resulted in an increase in the production of superoxide by the macrophages (Khajoee et al., 2005).

Summary

Johne's disease has a devastating impact on the dairy industry and is an issue of both economics and animal welfare. The studies presented in this dissertation focus on this destructive disease and the periparturient period. Despite on farm observations of progressing JD during the early post-parturient time period, there is a lack of research in the literature focusing on the host immune response to MAP at this time. In addition to characterizing cytokines and lymphocyte subsets during periparturient period, we also evaluated the potential role of Opn in regulating disease progression in MAP-infected periparturient cows. As reviewed in this chapter, Opn can enhance the Th1, cell-mediated, immune response and improve host defense against mycobacterial infections. To the best of our knowledge, this is the first known data investigating Opn gene and protein expression in MAP-infected cows. The experiments described in the subsequent chapters of this dissertation are guided by the hypothesis that the host immune response to MAP infection is altered during the periparturient period.

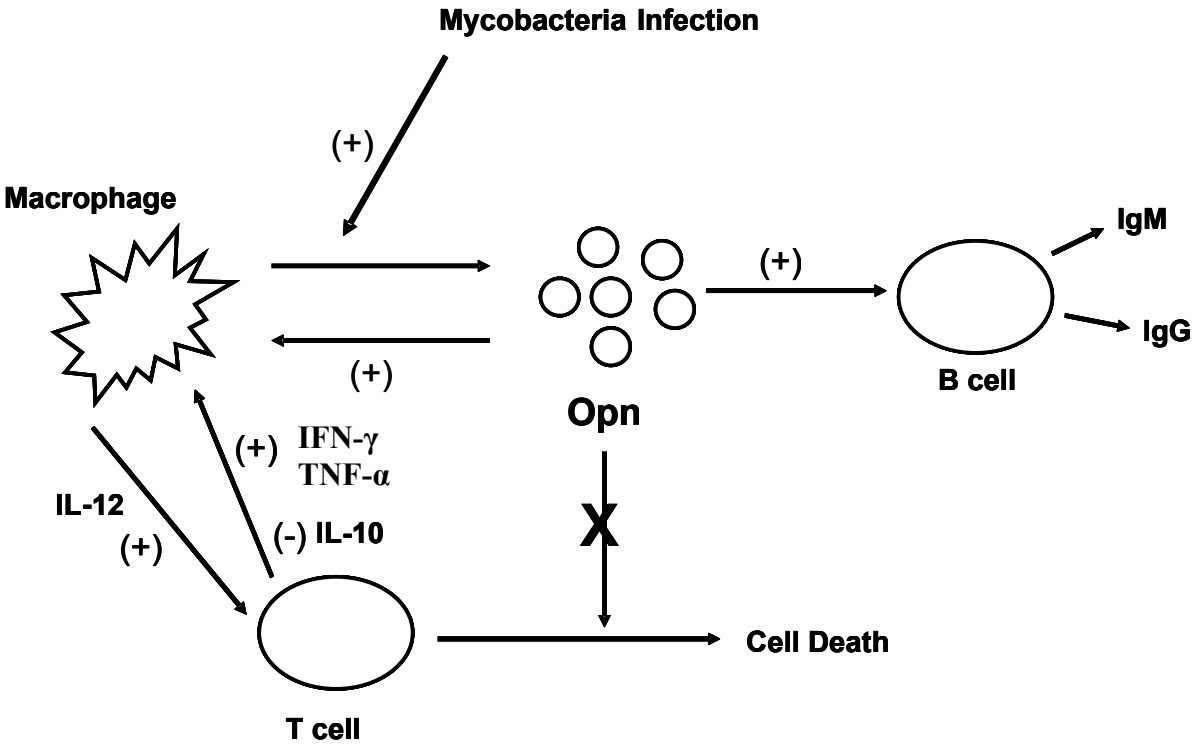

Figure 1.3. Role of osteopontin in regulating the Th1/Th2 balance in mycobacterial infection.

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CHAPTER TWO

MODULATION OF CYTOKINE GENE EXPRESSION AND SECRETION DURING THE PERIPARTURIENT PERIOD IN DAIRY COWS NATURALLY INFECTED WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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ABSTRACT

Johne's disease (JD) caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is estimated to infect more than 22% of US dairy herds. Periods of immunosuppression may contribute to the transition from the subclinical to clinical stage of infection. Understanding the effects of stressors such as parturition on the escalation of disease may provide information that will help manage JD. The objective of this study was to characterize cytokine gene expression and secretion in periparturient dairy cows naturally infected with MAP. Blood was collected from the jugular vein of healthy noninfected, and subclinically and clinically infected dairy cows for 3 wk pre- to 4 wk post-calving. Real-time PCR was performed to evaluate the expression of the following cytokine genes by peripheral blood mononuclear cells: IFN-γ, TNF-α, IL-12p35, IL-10, TGF-β, and IL-4. To assess the effects of parturient immunosuppression on cytokine gene expression, RT-PCR data were analyzed by using 2^{-ddCt} values calibrated to dCt value at +1 d relative to calving for each animal.

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Overall, cytokine gene expression was not influenced by infection status of the cows in this study. However, significant effects in cytokine gene expression were noted across sampling days within the periparturient period. Expression of IFN-γ, TNF-α, IL-4, and IL-10 declined at calving compared with prepartum values in both control and infected cows. PBMC isolated from infected cows secreted higher concentrations of IFN-γ, IL-10, and TGF-β in the postpartum period as compared to control cows. Clinically infected cows secreted higher levels of nitric oxide throughout the periparturient period when compared to control or subclinically infected cows. These data suggest that parturition is a very dynamic time period for host immunity, with potential for altered immunity to hinder the ability of dairy cows to thwart infectious diseases.

Key Words: periparturient, cytokine, *Mycobacterium avium* subsp. *paratuberculosis*

1. INTRODUCTION

Johne's disease (JD), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is estimated to infect more than 22% of US dairy herds and cost the US dairy industry $250 million annually (Ott et al., 1999). Dairy cows generally become infected as neonates through the fecal-oral route. Infected animals may remain in the subclinical, or asymptomatic, stage of the disease for several years following initial exposure of the bacteria (Larsen et al., 1975). During this stage, the majority of animals shed a negligible amount of bacteria, and granulomas begin to form in the small intestine. Stressors, such as parturition, may induce the transition from the subclinical to a more clinical stage of disease characterized by consistent fecal shedding of the bacteria, intermittent but persistent diarrhea,

progressive weight loss, and eventual death. Many questions still exist as to what triggers the progression of the disease.

Although it is anecdotal information, cows with paratuberculosis may advance to the clinical stage of the disease during the weeks following parturition. Research on what prompts the progression of disease during this time period is lacking. The transition period, defined as three weeks prior to and the three weeks following parturition, represents a time of physiological stress for the dairy cow (Goff and Horst, 1997). The immunosuppression experienced by the healthy periparturient cow has been well characterized. The week immediately following calving is marked by a decrease in lymphocyte proliferation (Kehrli et al., 1989b; Meglia et al., 2005) and the ability of neutrophils to migrate and phagocytize (Kehrli et al, 1989a; Lee and Kehrli, 1998). At calving, there is an increase in the activity of $CD8^+$ lymphocytes compared with those from mid to late lactation animals (Shafer-Weaver and Sordillo, 1997). Endocrine and metabolic changes (Goff and Horst, 1997), in combination with immune suppression, contribute to the susceptibility of the cow to both metabolic and infectious diseases. The dramatic changes in estrogen and progesterone in the periparturient period potentially play a key role in immune suppression. In periparturient dairy cows, progesterone concentrations decline rapidly at parturition and remain low during the initial weeks of lactation (Radcliff et al., 2003). Progesterone has the ability to up-regulate Th2 cell activity and contributes to natural suppression of cell-mediated immunity (Miyaura and Iwata, 2002). Plasma estrogen concentrations increase dramatically two weeks prior to calving and then decline rapidly at calving (Radcliff et al., 2003). Previous work has shown that estradiol enhances Th1 development *in vivo* (Maret et al., 2003). The decline in

progesterone and estradiol followed by decreased immune function may contribute to the progression of JD from subclinical to clinical state after calving.

The transition from the subclinical to the clinical stage of infection is characterized by a shift from cell-mediated (Th1) immunity to a Th2 response. Classical proinflammatory Th1 cytokines include IFN-γ, IL-12, and TNF-α (Mosmann et al., 1986; Roach et al., 2002). Expression of IL-12 promotes Th1 lymphocytes by inducing the secretion of IFN-γ (Bontkes et al., 2005) and subsequently enhancing the formation of granulomas (Smith et al., 1997). Th2 cytokines are predominantly considered to be suppressive and anti-inflammatory. Interleukin-4, IL-10, and TGF-β are capable of inhibiting Th1 cytokines, suppressing Th1 cell functions, and deactivating macrophages (Ho et al., 1992; Mullins et al., 2001).

A recent study demonstrated that nonstimulated PBMCs isolated from dairy cows with subclinical MAP infection had higher IFN-γ expression than did PBMCs from clinically infected cows or noninfected healthy controls (Coussens et al., 2004). Interestingly, in the same study, expression of IL-12 for infected animals was decreased compared to the controls. Recent data also demonstrated an increase in IFN- γ expression in subclinically infected cows (Khalifeh and Stabel, 2004b) and an upregulation of TGF-β and IL-10 in clinical cows naturally infected with MAP (Khalifeh and Stabel, 2004a). These studies provide further support for the paradigm of a Th1 response in early stages of JD with a switch to Th2-mediated responses as disease progresses.

To date, limited research is available characterizing host immunity in periparturient dairy cows infected with MAP or the potential impact of periparturient immunosuppression. Therefore, the objective of this study was to characterize cytokine gene expression and

secretion in dairy cows naturally infected with MAP during the periparturient period as compared with healthy control cows.

2. MATERIALS AND METHODS

2.1 Animals

Twenty-one multiparous Holstein cows and 2 primiparous Holstein cows were grouped according to infection status. These 3 groups consisted of 5 noninfected healthy cows, 14 cows naturally infected with MAP, but asymptomatic, and 4 naturally infected cows with clinical Johne's disease. The 2 primiparous cows were both in the subclinically infected group. The stage of infection was determined by fecal shedding of MAP, IFN-γ secretion, and specific antibody response to MAP. Infection was monitored by bacteriologic culture for the fecal shedding of MAP by standard methods (Stabel, 1997). By definition, clinical animals were shedding more than 100 CFU/tube (BBL™ Herrold's Egg Yolk Agar Slants with mycobactin J, amphotericin, nalidixic acid, and vancomycin; Becton, Dickinson and Co., Sparks, MD) and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 CFU/tube and were asymptomatic. The noninfected control cows were characterized by repeated negative fecal cultures performed quarterly over a 3- to 5-yr period and had been purchased from herds with no recent history of Johne's disease. In addition, these animals were negative on any serologic assays (i.e., production of antibody specific for MAP and IFN-γ) performed during that period. All procedures performed on the animals were approved by the Institutional Animal Care and Use Committee (National Animal Disease Center [NADC], Ames, IA.

2.2 Blood Collection, Culture Conditions, and Sample Collection

Blood was collected from the jugular vein in 2x acid-citrate-dextrose (ACD; 1:10). For each animal, blood was collected at -21, -14, -7, +1, +7, +14, +21, and +28 days relative to calving. Peripheral blood mononuclear cells were isolated from the buffy coat fractions of peripheral blood. PBMCs were resuspended in RPMI-1640 (Gibco, Grand Island, NY) with 10% fetal calf serum (Atlanta Biologics, Atlanta, GA), 100 U of penicillin G sodium per ml, 100 μg of streptomycin sulfate per ml, 0.25 μg of amphotericin B per mL, and 2 m*M L*-glutamine (Gibco, Grand Island, NY).

Cells were cultured at 1.4 x 10^6/mL in 48-well flat-bottomed plates (Corning, Corning, NY) with either medium alone (nonstimulated**,** NS), with ConA (10 μg/mL) or with MAP whole cell sonicate (MPS; 10 μg/mL) added to designated wells. Plates were incubated for 24 h at 39°C in 5% CO_2 in a humidified atmosphere. After 24 h plates were removed and centrifuged at 400 x g for 5 min. Supernatants were removed without disturbing the cells in culture, and stored at -20°C prior to cytokine measurement.

Additional blood samples were collected at each time point into vacutainer tubes (Becton Dickinson, Rutherford, NJ), and serum was harvested and stored at -20°C. Serum progesterone and 17β-estradiol were quantified by using double antibody radioimmunoassay kits with the human standards supplied (ImmuChem™ Double Antibody, MP Biomedicals, Costa Mesa, CA). Serum insulin-like growth factor I (IGF-I) was quantified by using a non-extraction two-site immunoradiometric assay (DSL-2800; Diagnostic Systems Laboratories, Inc., Webster, TX). The minimal sample concentration of IGF-1 that could be detected by the kit was 9 ng/mL. The intra-assay coefficient of variation for progesterone was 4.14% and the interassay variation was 12.8%. For 17β-estradiol, the intra-assay coefficient of variation

was 4.10%, and the interassay variation was 10.00%. The intra-assay coefficient of variation for IGF-1 was 5.08%, and the interassay variation was 4.48%.

2.3 Bacteria

M. avium subsp. *paratuberculosis* strain K-10 (NADC) was grown in Middlebrook 7H9 broth (pH 6.0) supplemented with mycobactin J (2 mg/liter; Allied Monitor, Fayette, MO) and oleic acid-albumin-dextrose complex (Becton Dickinson Microbiology, San Jose, CA). The bacteria were harvested, washed 3 times with PBS (137 m*M* sodium chloride, 10 m*M* phosphate, 2.7 m*M* potassium chloride; pH 7.4), and sonicated on ice for 10 min. After incubating at RT for 10 min, the bacteria was sonicated for an additional 10 min and then centrifuged at 2500 x g for 20 min. Supernantant was removed and absorbance was read at 540 nm. Bacterial stocks (10^9/mL) were frozen in PBS at -80°C until used in the experiments.

2.4 RNA Extraction and RT-PCR

Peripheral blood mononuclear cells from each of the sampling time points were resuspended in RPMI 1640 (Gibco, Grand Island, NY) containing 10% fetal calf serum. PBMCs from each cow at each sampling time point were split into two aliquots: one aliquot that was cultured with medium alone (NS) and one that was stimulated with ConA (10 μg/mL). Cells were cultured in 75-cm^2 flasks at a concentration of 1 x 10^7 PBMCs per flask at 39°C in 5% CO_2 in a humidified atmosphere for 24 h. RNA was extracted from NS and ConA-stimulated PBMCs by using the standard protocol for Trizol Reagent (Invitrogen Life Technologies Corp., Carlsbad, CA). All RNA samples were purified by using the RNeasy® Mini Kit Protocol for RNA Cleanup (Qiagen, Valencia, CA). Samples were treated with

TURBO DNA-free (Ambion, Austin, TX). The quantity of total RNA was determined by UV spectrophotometry. RNA samples were frozen at -80°C until converted to cDNA.

Real time RT-PCR was performed by using an Applied Biosystems 7500 DNA sequence detection system (Perkin-Elmer Corp., Foster City, CA). Total RNA extracted from both NS and ConA-stimulated cells was converted to first strand cDNA. Briefly, 2 μg of total RNA was added to 12-μl reaction mixture consisting of 10 m*M* oligo$(dT)_{12\text{-}18}$ primer (Invitrogen, Carlsbad, CA) and RNAse-free water. The reaction mixture was incubated at 70°C for 5 min and then quickly chilled on ice to 20°C. To the reaction mixture, 4 μl of 5x First Strand Buffer (Invitrogen, Carlsbad, CA), 2 μL of 10 m*M* dNTP Mix (Invitrogen, Carlsbad, CA), 1 μL of 0.1 *M* dithiothreitol (Invitrogen, Carlsbad CA), and 2 U SuperScript™ RNase H-Reverse Transcriptase (Invitrogen, Carlsbad CA), were added for a total volume of 20 μL. The reaction mixture was incubated at 42°C for 1 h, heated to 70°C for 15 min, and then cooled to 37°C. Two units of DNase-free RNase H (Invitrogen, Carlsbad, CA) was added to the mixture that then was incubated at 37°C for 20 min to remove the original RNA template. The RNase H was inactivated by heating the reaction mixture at 70°C for 10 min. All cDNA samples were stored at -80°C until RT-PCR analyses were performed.

For RT-PCR analysis, SYBR Green PCR master mixture (Perkin-Elmer Corp., Foster City, CA), template cDNA, and gene-specific primers for IFN-γ, TNF-α, IL-12p35, IL-4, IL-10, TGF-β, and β-actin were combined in a 20 μL reaction mixture. Primer sequences are listed in Table 1. All reactions were performed in triplicate. The β-actin gene was used as the control for calculation of dCt. RT-PCR data were analyzed by using the $2^{-(ddCt)}$ method

as described previously (Livak and Schmittgen, 2001). The mean +1 DRTC dCt value within treatment was used as the reference expression point.

2.5 Measurement of IFN-γ, IL-10, TGF-β, and Nitric Oxide Production in Cell Culture Supernatants by ELISA

Bovine IFN-γ was measured by using the Bovigam test kit (Biocor Animal Health, Omaha, NE) as described by the manufacturer. The minimal sample concentration of IFN-γ that could be detected by using the kit was 0.39 ng/mL. Bovine IL-10 was quantified by coating MaxiSorp microtiter plates (Nunc, Rochester, N.Y.) with mouse anti-bovine IL-10 in coating buffer (15 m*M* sodium carbonate, 34 m*M* sodium bicarbonate, pH 9.6) (100 µL per well at 2 µg/mL) (MCA2110, Serotec, Raleigh, NC) overnight at RT. Plates were washed 5 times with PBS containing 1% Tween 80 (washing buffer). The samples and serial 2-fold dilutions of bovine IL-10 standard (0.3125-20 ng/mL) (generous gift from Dr. Jayne Hope, Compton, UK) were added to duplicate wells and incubated at RT for 1 h. Plates were then washed 5 times with washing buffer before incubating with the detection antibody, mouse anti-bovine IL-10:biotin (MCA2111B, Serotec, Raleigh, NC). Plates were washed 5 times with washing buffer, 100 µL of avidin-HRP conjugate (diluted 1:800) (PharMingen, San Diego, CA) was added to each well, and the plates were incubated for 45 min at RT. After another wash cycle, plates were incubated with substrate solution (40 mM ABTS [2, 2'-azino-di-ethylbenzthiozoline-6-sulfonic acid] in citrate buffer (50 m*M*, pH 4.0) and H_2O_2 (30% solution as 1:30 dilution). Color development was quantified after 30 min by measuring absorbance at 405 nm with a Wallac Victor 1420 multilabel counter ELISA plate

reader (Perkin-Elmer, Gaithersburg, MD). The minimal sample concentration of IL-10 that could be detected by using the kit was 0.3125 units.

Bovine TGF-β was quantified using the Quantikine® Human TGF-β1 Immunoassay kit as described by the manufacturer and by using standards supplied (R&D Systems, Inc, Minneapolis, MN). Previous studies have utilized anti-bovine TGF-β1 antibodies to detect bovine TGF-β activity (Ginjala and Pakkanen, 1998; Khalifeh and Stabel, 2004a). Significant concentrations of latent TGF-β1 are found in bovine cell culture supernatants. Latent TGF-β was activated by the addition of 1 N HCl followed by 10 min of incubation. The samples were neutralized by the addition of 1.2 N NaOH/0.5 M HEPES. The concentration determined by the standard curve was multiplied by the dilution factor 1.4 to account for the activation procedure. Because of the high cost of the kit and large number of samples in this study, only NS and MPS-stimulated cell culture supernatants were evaluated at -14, -7, +1, +7,+14, and +21 days for this assay. The minimal sample concentration of TGF-β that could be detected by using the kit was 31.2 pg/mL.

To determine the amount of nitric oxide produced (NO), the stable oxidation product, nitrite, was quantitated. For this analysis, 24 h cell culture supernatants from NS, ConA, and MPS-stimulated PBMCs were evaluated at -21, -14, -7, +1, +7, +14, +21, and +28 days. The samples and serial two-fold dilutions of sodium nitrate standard (0.028-18 μM) were added in duplicate to a 96 well- round bottom plate (Corning, Corning, NY). The culture supernatant (100 μl) was mixed with 100 μl of Greiss reagent (0.5% sulfanilamide; Sigma Chemical Co., St. Louis, MO) in 2.5% phosphoric acid (Mallinckrodt Chemical, Inc., Hazelwood, MO) and 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma Chemical Co., St. Louis, MO). The mixture was allowed to incubate at RT for 10 min, and

color development was measured at 505 nm. The concentration of nitrite in the supernatant was quantified by comparison with absorbance values of sodium nitrite standards within a linear curve fit. The minimal sample concentration of NO that could be detected using the kit was 0.28 μM.

2.6 Statistical Analysis

RT-PCR data were analyzed by using the $2^{-(ddCt)}$ method as previously described (Livak and Schmittgen, 2001). β-actin was used as the reference gene, and the dCt value at +1 d for each animal was used as the reference expression point. Outliers were determined by the SAS/STAT PROC RobustReg software. To evaluate the appropriateness of β-actin as a reference gene, the $2^{-(dCt)}$ method was used as previously described (Schmittgen and Zakrajsek, 2000). This test indicated that β-actin would serve as a suitable reference for healthy and MAP-infected animals.

Several 2-factor repeated measures analyses of variance were performed (one for each stimulation level-gene combination) comparing fold expression of 2 treatments through time. Levene's homogeneity of variance test was performed on data to determine transformation necessity. All analyses were performed on transformed data where necessary, but raw data means are presented for ease of interpretation. If a significant F-test value from the ANOVA was obtained at $P \leq 0.05$, differences of least squares means were used as the pairwise multiple comparison test for determining day or infection group x day differences. Means differed if $P < 0.05$ and tended to differ if $0.05 \leq P \leq 0.15$. Analyses were performed using PROC MIXED in SAS® PC Windows Version 9.1.3 software.

3. RESULTS

3.1 Effect of infection status on cytokine gene expression

In this study, +1 day was used as the reference expression point in the analyses of the gene expression data. Due to sampling error, 3 of the 4 clinical cows did not have useable RNA samples for the +1 d time point. Because of the nature of the periparturient study, we were not able to retrospectively isolate additional PBMCs from the blood of these cows at +1 d for RNA extraction. In the 2^{-ddCt} analysis, it is critical to have the dCt for the reference expression point day. On the basis of these results, we were not able to evaluate the cytokine gene expression data for the clinical cows in this study. All gene expression data presented herein contrasts results between healthy noninfected control cows and subclinically infected cows.

Regardless, there were no significant effects due to infection status on the expression of cytokines, IFN-γ, IL-12p35, TNF-α, IL-4, IL-10, and TGF-β during the period extending from 3 wk prior to calving to 4 wk post-calving (Figs. 1-2). There was also no effect of infection group on expression of IGF-1 from NS PBMCs (Fig.3), however, a strong trend was present demonstrating higher expression of IGF-1 for PBMCS isolated from subclinically infected cows compared with that of control cows in the prepartum period.

3.2 Effect of parturition on cytokine gene expression

There was a significant effect of parturition on IFN-γ expression in NS ($P < 0.05$) and ConA-stimulated ($P < 0.01$) PBMCs (Fig. 1A). For both groups, expression declined beginning on -14 d through calving. Expression in NS PBMCs did not recover during the

postpartum period. However, IFN-γ expression by ConA-stimulated PBMCs was significantly ($P < 0.01$) greater by +28 d compared with -7 d (Fig. 1B).

In the NS PBMCs, expression of TNF-α was variable throughout the periparturient period, but declined for both groups of cows from -7 d to +1 d and rebounded by +7 d ($P < 0.01$) (Fig. 1C). Despite the lack of an overall effect of parturition on ConA-stimulated PBMCs, TNF-α expression significantly ($P < 0.05$) declined from +1 d to +21 d for control cows (Fig. 1D).

Similarly, there was no effect of parturition on IL-12p35 or TGF-β expression for either NS or ConA-stimulated PBMCs (data not shown). Expression of both cytokines remained relatively stable during the 3 wk before and 4 wk after calving, regardless of infection group.

A significant ($P < 0.05$) effect of parturition on IL-10 expression was noted for the NS PBMCs (Fig. 2A). During the prepartum period, all cows exhibited a decline in IL-10 expression ($P < 0.05$) between d -21 and calving, but control cows showed an increase ($P < 0.01$) in IL-10 expression during the postpartum period. Stimulating the PBMCs with ConA did not result in overall effects of parturition on IL-10 mRNA expression (Fig. 2B); however, there was a decline in expression between -21 d and -7 d in subclinical cows ($P < 0.05$). There was no effect of parturition on IL-4 expression by NS PBMCs (Fig. 2C). A linear decrease in IL-4 expression was noted for ConA-stimulated PBMCs isolated from subclinically infected cows between -21 d and +1 d ($P < 0.05$) (Fig. 2D).

For both control and subclinical animals, there was a significant increase in IGF-1 expression at parturition followed by a sharp decline post-calving ($P < 0.001$) (Fig. 3).

3.3 IFN-γ, IL-10, TGF-β, and Nitric Oxide Secretion

There was no effect of infection or parturition on secretion of IFN-γ from NS PBMCS. Overall, throughout the sampling period, production of IFN-γ by ConA-stimulated PBMCs tended to be greater in subclinical (14.36 ng/ml ± 1.6; $P < 0.06$) and clinical (14.28 ± 2.7; $P < 0.12$) cows than the controls (8.30 ± 2.2) (Fig. 4A). There was an overall effect of parturition and an interaction of infection group and parturition ($P < 0.0001$), but this effect was most likely the result of extremely high concentration of ConA-stimulated IFN-γ noted on -21 d for the clinical cows. By -14 d, this value was within the range of the average values noted for subclinical and control cows. A significant ($P < 0.05$) increase in ConA-stimulated IFN-γ secretion was observed for subclinically infected cows on d +21 and +28 post-calving as compared to other groups. When PBMCs were stimulated with MPS, there was a tendency ($P < 0.11$) for clinical cows to secrete more IFN-γ compared with the controls (Fig. 4B). A significant ($P < 0.001$) decline in MPS-stimulated IFN-γ secretion was observed for clinical cows from -21 d (2.18 ng/mL ± 0.8) to +1 d (0.75 ng/mL ± 0.2. Concentrations for subclinical and control cows remained relatively stable throughout the sampling period.

Overall, secretion of IL-10 by NS and ConA-stimulated PBMCs was not affected by infection group (Fig. 5). There was a spike in IL-10 secretion in the prepartum period by NS PBMCs from subclinical cows with secretion increasing sharply from -21 d to -14 d and then rapidly declining to +1 d ($P < 0.01$) (Fig. 5A). Clinical cows followed a similar pattern with secretion increasing sharply from -14 d to -7 d and then declining to a nadir of 0.307 U at +1 d. This resulted in an overall interaction of infection group and parturition ($P < 0.001$). When PBMCs were stimulated with ConA, there was no longer an effect of parturition (data

not shown). For the MPS-stimulated PBMCs, the days were combined into three periods: -21 d, -14 d, -7 d = precalving; +1 d = calving; and +7 d, +14 d, +21 d = postcalving. Also, because of similar trends for both infected groups, subclinical and clinical cows were combined into one infected group. Secretion of IL-10 tended ($P < 0.09$) to be greater for infected cows compared with that of control cows at calving and during the postpartum period (Fig. 5B). Interestingly, stimulating the PBMCs with MPS resulted in a 7.7-, 9.7-, and 12.0-fold increase in secretion for control, subclinical, and clinical cows, respectively, compared with secretion from NS PBMCs (data not shown).

Overall, parturition did not seem to have a significant effect on TGF-β production by PBMC's stimulated with medium only (NS) (Fig. 5C). However, at +7 d, subclinical cows had significantly lower TGF-β secretion compared with control cows ($P < 0.05$; 868.5 ± 79.3 pg/mL vs. 1187.3 ± 77.9 pg/mL) and clinical cows ($P < 0.01$; 868.5 ± 71.1 pg/mL vs.1305.6 ± 98.9 pg/mL) at +7 d. In addition, an overall trend for higher TGF-β secretion by NS PBMC isolated from clinical cows was noted in the postpartum period. There was no overall effect of cow infection status on concentrations of TGF-β when PBMCs were stimulated with MPS, except at +1 d when clinical cows had significantly ($P < 0.05$) increased TGF- β secretion compared with that of subclinical and control cows (Fig. 5D).

Nitric oxide production by NS PBMCs was not affected by either infection group or parturition (data not shown). In the MPS-stimulated cells, the clinical cows (0.75 μM ± 0.2) tended to have greater NO production compared with that of the control (0.31 μM ± 0.15; $P < 0.10$) and the subclinical cows (0.42 μM ± 0.09; $P < 0.15$), and an overall infection group x parturition interaction was noted ($P < 0.10$) (Fig. 6B). Similar effects were observed for ConA-stimulated cells, but the effects were not significant (Fig. 6A).

3.4 Serum Progesterone, 17β-Estradiol, and IGF-1

For all cows sampled, regardless of infection status, progesterone concentrations declined as parturition approached (Fig. 7A). This decline resulted in a strong day effect ($P < 0.001$). At -21 d, subclinical cows showed significantly ($P < 0.05$) elevated progesterone compared with control cows. During the sampling days after calving, concentrations of serum progesterone declined and reached values lower than that that could be detected by using the kit (0.2 ng/mL). Estradiol concentrations remained fairly constant for control and clinically infected cows until -7 day (Fig. 7B). At d -7, estradiol concentrations began to decline and by d +7 reached a nadir of 10 pg/mL. This decline was characteristic for all cows, regardless of infection status, resulting in an overall day effect ($P < 0.001$). At -7 d, estradiol spiked for subclinically infected cows with concentrations greater than both control ($P < 0.001$) and clinical ($P < 0.001$) cows and declined thereafter.

In general, for healthy control cows and infected cows, IGF-1 concentrations declined between -14 d and +1 d and then began to recover after parturition (Fig. 7C). The decline in the weeks prior to parturition and subsequent recovery of IGF-1 serum concentrations within the first few weeks after parturition resulted in an overall day effect ($P < 0.0001$). There was no overall effect of MAP infection on serum IGF-1 concentrations.

4. DISCUSSION

Johne's disease has a devastating impact on the health of the dairy cow, and this disease has had cumulative economic impact on the dairy producer. Most animals will encounter MAP during the first year of life and will remain in the subclinical, or

asymptomatic, stage of the disease for several years. The transition from subclinical to clinical state coincides with the differentiation of naïve $CD4^+$ T-cells from a Th1 to a Th2 immune response. Limited research exists that focuses on the role that parturition plays, if any, in the progression of JD from the subclinical to clinical state. To the best of our knowledge, this study is the first to document changes in cytokine gene expression and secretion in dairy cows naturally infected with MAP throughout the periparturient period.

Even the healthy dairy cow experiences increased challenges to the immune system during the periparturient period. Increases in both metabolic and infectious disease are observed during this time (Goff and Horst, 1997). Throughout mid to late lactation, cytokine profiles mirror a Th1 immune response. However, as transition to lactation occurs, there is a shift in the $CD4^+$ population supporting a Th2 response (Shafer-Weaver et al., 1999). The immune system is further compromised by decreases in both neutrophil and lymphocyte function that is caused by parturition (Kehrli et al., 1989a; Kehrli et al., 1989b).

To better understand the interaction of parturition and MAP infection, we evaluated the ability of cytokines to modulate progression of JD during the periparturient period by first examining the expression of the Th1 cytokines. In the current study, MAP infection did not have an effect on the gene expression of IFN-γ, TNF-α, or IL-12. These results are in contrast to previous work in our laboratory that concluded that subclinical JD cows have greater IFN-γ expression compared with that of clinical cows (Stabel, 2000; Khalifeh and Stabel, 2004a; Khalifeh and Stabel, 2004b). However, PBMCs from subclinical cows did tend to secrete more IFN-γ compared with clinical and control cows. Interferon-γ is important for initiating the innate immune response to intracellular bacteria and it is one of the first cytokines to be activated in subclinical MAP-infected dairy cows (Sweeney et al.,

1998). Together, IFN-γ and TNF-α promote the protective formation of granulomas (Roach et al., 2002), a critical step in controlling MAP-infection. Both IFN-γ and TNF-α expression by NS PBMCs declined as parturition approached, which is in agreement with previous observations suggesting a decline in Th1 cytokines at parturition (Shafer-Weaver et al., 1999). Interestingly, IL-12 expression was not affected by infection or parturition. This is in contrast to Coussens et al. (2004) who showed that PBMCs from MAP-infected cows expressed 13-fold less IL-12p35 than did cells from control cows.

In addition to understanding the effects of parturition and MAP infection on the Th1 cytokines, we also sought to determine the role of the classical Th2 cytokines, IL-4 and IL-10, and the Th3 cytokine, TGF-β. The transition from the Th1 to Th2 represents a switch from cell-mediated to antibody-mediated immunity. In the current study, there was no effect of parturition or MAP infection on gene expression of the Th2 or Th3 cytokines. Recent data from our laboratory suggested that there was an upregulation of TGF-β and IL-10 expression in clinical cows naturally infected with MAP (Khalifeh and Stabel, 2004a). TGF-β has been shown to act synergistically with IL-10 in repressing macrophage activation (Mullins et al., 2001). Despite minimal differences in TGF-β expression, NS and MPS-stimulated PBMCs isolated from clinical cows secreted more TGF-β during the immediate postpartum period compared with subclinical and control cows. MPS-stimulated PBMCs from infected cows secreted more IL-10 than did the control cows at calving and during the postpartum period. The increase in IL-10 secretion observed during the postpartum period for infected cows may result in the down-regulation of macrophage and natural killer cell function. Interleukin-4 functions to promote B-cell activation and Th1 cell suppression. Others have reported

upregulation of IL-4 during the immediate postpartum period but this effect was not observed in the present study (Shafer-Weaver and Sordillo, 1997; Shafer-Weaver et al., 1999).

It is of interest to note that the cytokine profiles for the cows in our study typically followed the established Th1/Th2 dichotomy observed in periparturient dairy cows. We did not, however, observe an effect of MAP infection on cytokine expression. Both the innate and adaptive immune responses are lowest during the periparturient period (Mallard et al., 1998). To date, there is not a complete characterization of the consequences of this immunosuppression. There exists a possibility that the immunosuppression at calving was severe enough to essentially override the traditional Th1/Th2 paradigm observed in MAP-infected animals. This may explain why we did not observe differences in cytokine expression between infection groups.

Hormonal fluctuations during the periparturient period contribute to the typical immunosuppression observed at calving. The polarization of a specific Th response may be influenced by the hormonal status of the animal. On the basis of their potential role during this critical time period, we evaluated IGF-1, progesterone, and 17β-estradiol concentrations in the peripheral blood. All of the cows exhibited the typical hormonal responses observed around parturition (Radcliff et al., 2003). Despite our hypothesis, MAP infection did not appear to affect blood hormone concentrations.

One major hypothesis of the current study was that subclinical cows would transition to the clinical stage of the disease during the 4 wk postpartum. However, not one of the 14 subclinical cows on the study seemed to make the transition to the clinical stage of the disease during the 4 wk postpartum. This conclusion was based on diagnostic measurements such as fecal shedding of the bacteria, antigen-specific IFN-γ responses, and ELISA titers for

the entire sampling period. Further studies are needed to determine if the cytokine profile of JD cows is altered later in lactation than the +28 d observed in the current study.

5. CONCULSIONS

Results of this study indicate that parturition modulates mRNA expression of IFN-γ, TNF-α, IL-10, and IGF-1 in dairy cows. Infection with MAP did not significantly alter cytokine gene expression compared with that of control cows, but secretion of IFN-γ, IL-10, and TGF-β were up-regulated by infection in the postpartum period. The periparturient period did not alter the transition from subclinical to clinical infection status in cows with paratuberculosis, but the period of observation was relatively brief. A more protracted period of study would likely yield more significant effects. In addition, the highly dynamic nature of the immediate periparturient period may have precluded distinct observations between infection groups.

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Table 1. Primers used for RT-PCR of bovine cytokines

Gene	Primer[1]	Sequence
β-actin	F	CGCCATGGATGATGATATTGC
β-actin	R	AAGCCGGCCTTGCACAT
IL-4	F	GCCACACGTGCTTGAACAAA
IL-4	R	TGCTTGCCAAGCTGTTGAGA
IL-10	F	GCCTTGTCGGAAATGATCCA
IL-10	R	TCAGGCCCGTGGTTCTCA
IL-12p35	F	CTTTCTTCAAATGCAGCATTGG
IL-12p35	R	GGGTCTGGGTGATACAACGAA
IFN-γ	F	AGCCAAATTGTCTCCTCCTACTTC
IFN-γ	R	CTGACTTCTCTTCCGCTTTCTG
TNF-α	F	CAAGTAACAAGCCGGTAGCC
TNF-α	R	TGGAAGACTCCTCCCTGGTA
TGF-β	F	CTGAGCCAGAGGCGGACTAC
TGF-β	R	TTGCTGAGGTAGCGCCAGGAATTG

[1]F, forward; R, reverse.

Figure Legends

Figure 1. Expression of Th1 cytokines, IFN-g and TNF-a, by peripheral blood mononuclear cells from healthy control cows (♦) and cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (■). **A)** IFN-γ expression by nonstimulated (NS) PBMCs. (parturition effect, $P < 0.05$). **B)** IFN-γ expression by concanavalinA-stimulated PBMCs. (parturition effect, $P < 0.001$). **C)** TNF-α expression by NS PBMCs. (parturition effect, $P < 0.01$). **D)** TNF-α expression by concanavalinA-stimulated PBMCs. (parturition effect, $P < 0.05$). Significant differences on a given day relative to day 1 are represented by asterisks ($P < 0.05$).

Figure 2. Expression of Th2 cytokines, IL-10 and IL-4, by peripheral blood mononuclear cells from healthy control cows (♦) and cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (■). **A)** IL-10 expression by nonstimulated (NS) PBMCs. (parturition effect, $P < 0.05$). **B)** IL-10 expression by concanavalinA (ConA)-stimulated PBMCs. **C)** IL-4 expression by NS PBMCs. **D)** IL-4 expression by ConA-stimulated PBMCs. Significant differences on a given day relative to day 1 are represented by asterisks ($P < 0.05$).

Figure 3. Insulin-like growth factor-1 mRNA expression by non-stimulated peripheral blood mononuclear cells from healthy control cows (♦) and cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (■). (parturition effect, $P < 0.0001$). Significant differences on a given day relative to day 1 are represented by asterisks ($P < 0.05$).

Figure 4. Interferon gamma secretion by peripheral blood mononuclear cells isolated from control (♦), subclinical (■), and clinical (▲) periparturient dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. **A)** ConcanavalinA-stimulated PBMCs. (parturition effect, $P < 0.0001$; infection x parturition effect, $P < 0.001$). **B)** MAP whole cell sonicate-stimulated PBMCS. (parturition effect, $P < 0.05$; infection x parturition effect, $P < 0.01$). Significant differences between infection groups on a given day relative to calving are represented by asterisks ($P < 0.05$).

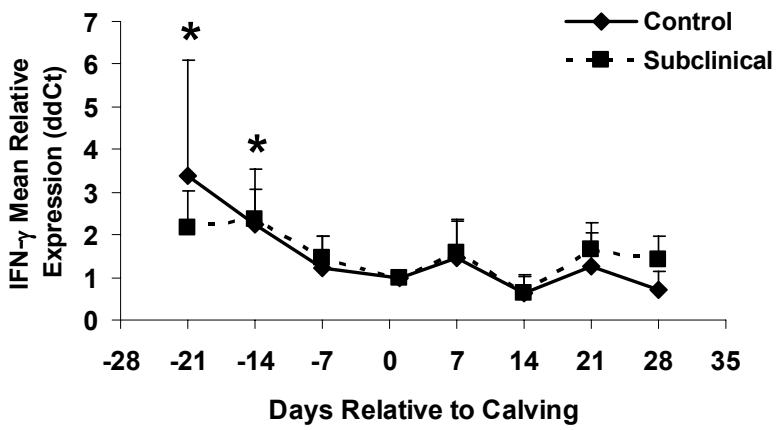
Figure 5. Interleukin 10 and transforming growth factor beta secretion by peripheral blood mononuclear cells isolated from control (♦), subclinical (■), and clinical (▲) periparturient dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. **A)** IL-10 secretion by nonstimulated (NS) PBMCs. (parturition effect, $P < 0.01$; infection x parturition effect, $P < 0.001$). **B)** IL-10 secretion by MAP whole cell sonicate (MPS)-stimulated PBMCs. **C)** TGF-β secretion by NS PBMCs. **D)** TGF-β secretion by MPS-stimulated PBMCs. Significant differences between infection groups on a given day relative to calving are represented by asterisks ($P < 0.05$).

Figure 6. Nitrite production by peripheral blood mononuclear cells isolated from control (♦), subclinical (■), and clinical (▲) periparturient dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. Nitrite is the stable product of nitric oxide. The amount of nitrite in the sample is proportional to the amount of nitric oxide secreted by the PBMCs. **A)** ConcanavalinA-stimulated PBMCs. (parturition effect $P < 0.05$). **B)** MAP whole cell sonicate-stimulated PBMCs. (parturition effect, $P < 0.01$). Significant differences between infection groups on a given day relative to calving are represented by asterisks ($P < 0.05$).

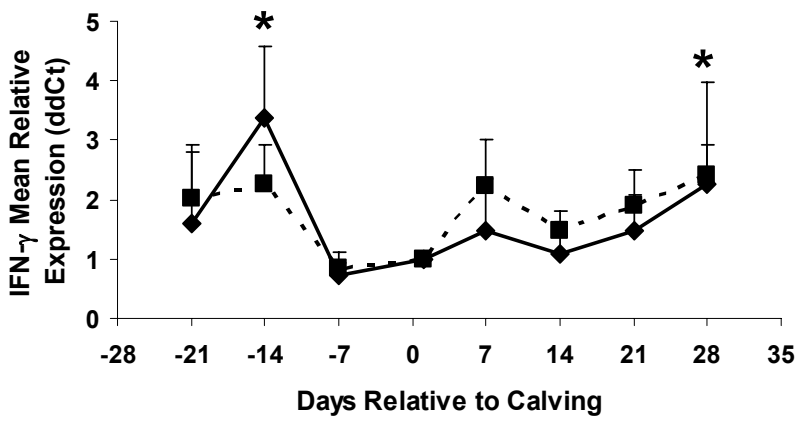
Figure 7. Serum hormone concentrations obtained from peripheral blood of control (♦), subclinical (■), and clinical (▲) periparturient dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. **A)** Progesterone concentration. (parturition effect, $P < 0.001$). **B)** 17β-estradiol concentration. (parturition effect, $P < 0.0001$). **C)** IGF-1 concentration. (parturition effect, $P < 0.0001$). Significant differences between infection groups on a given day relative to calving are represented by asterisks ($P < 0.05$).

Figure 1.

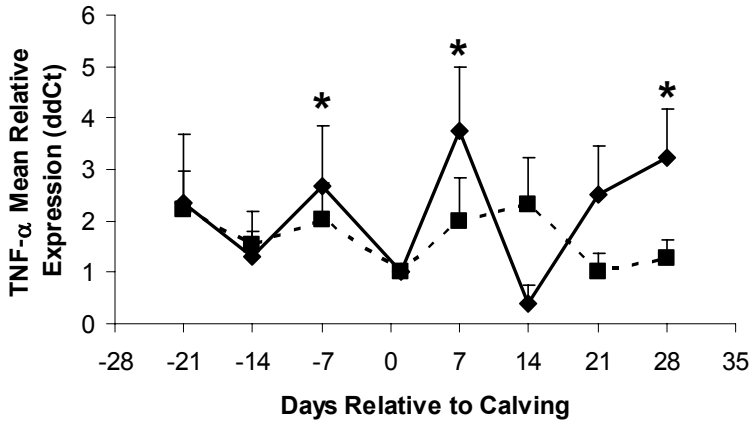
A.



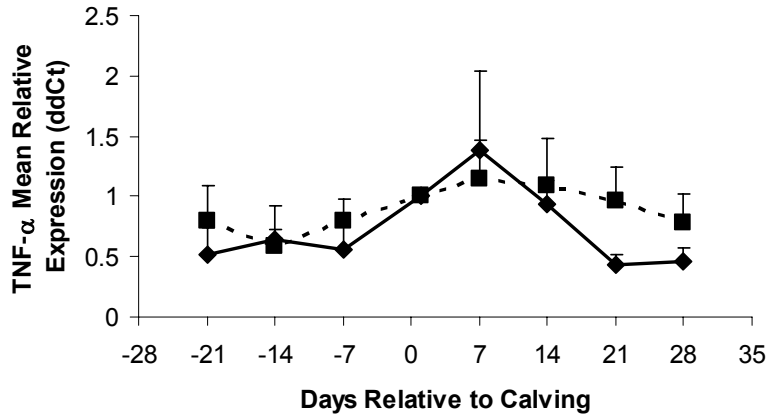
B.



C.



D.



♦ Control ■ Subclinical

Figure 2

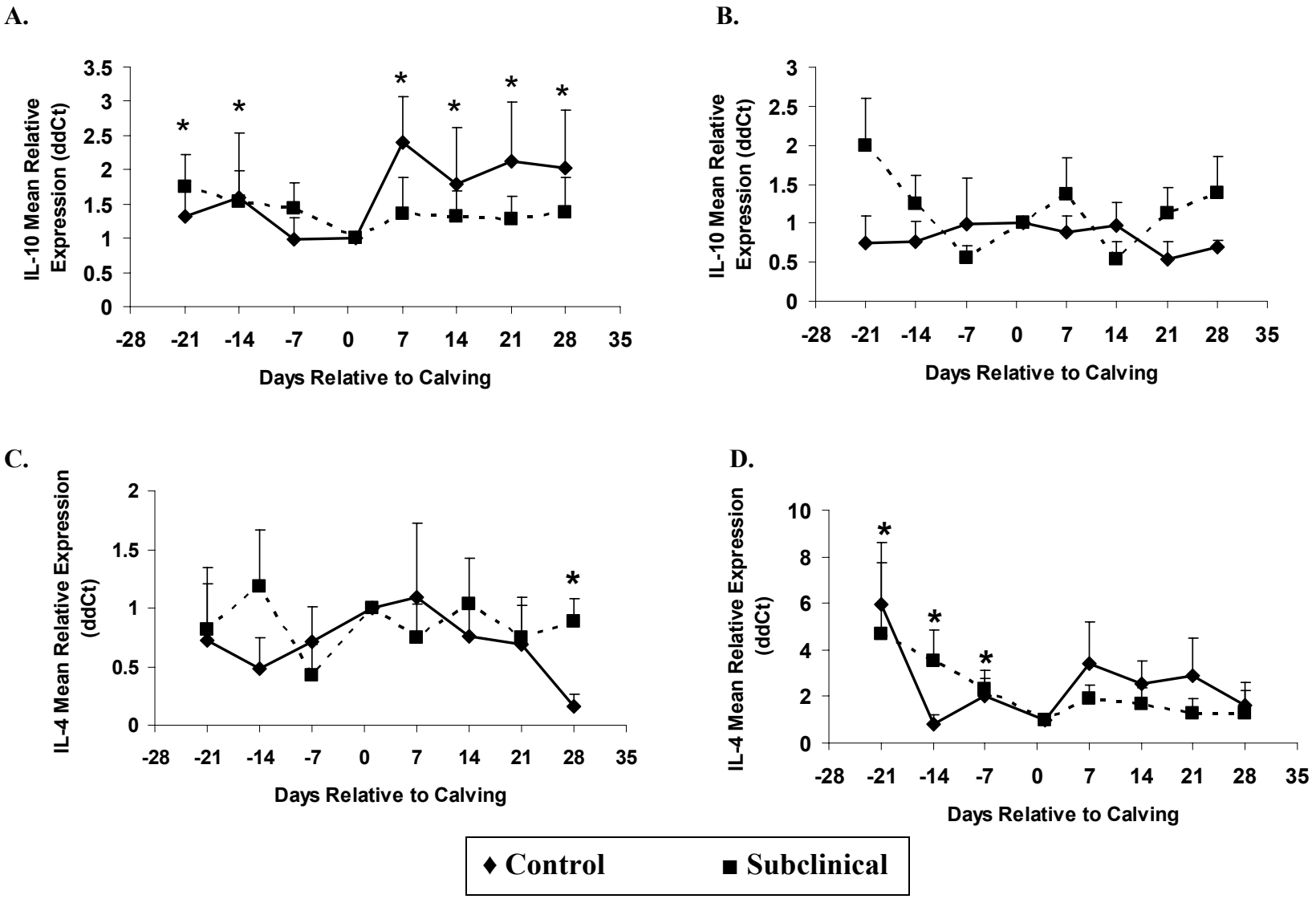

A.
IL-10 Mean Relative Expression (ddCt)
3.5
3
2.5
2
1.5
1
0.5
0
-28 -21 -14 -7 0 7 14 21 28 35
Days Relative to Calving
B.
IL-10 Mean Relative Expression (ddCt)
3
2.5
2
1.5
1
0.5
0
-28 -21 -14 -7 0 7 14 21 28 35
Days Relative to Calving
C.
IL-4 Mean Relative Expression (ddCt)
2
1.5
1
0.5
0
-28 -21 -14 -7 0 7 14 21 28 35
Days Relative to Calving
D.
IL-4 Mean Relative Expression (ddCt)
10
8
6
4
2
0
-28 -21 -14 -7 0 7 14 21 28 35
Days Relative to Calving
♦ Control
■ Subclinical

Figure 3.

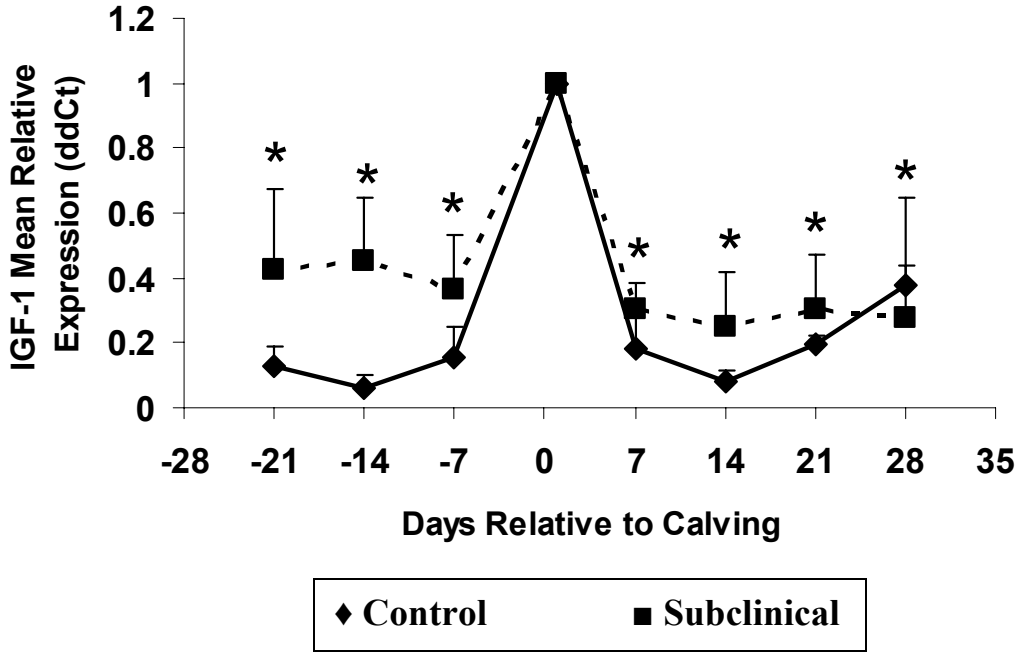
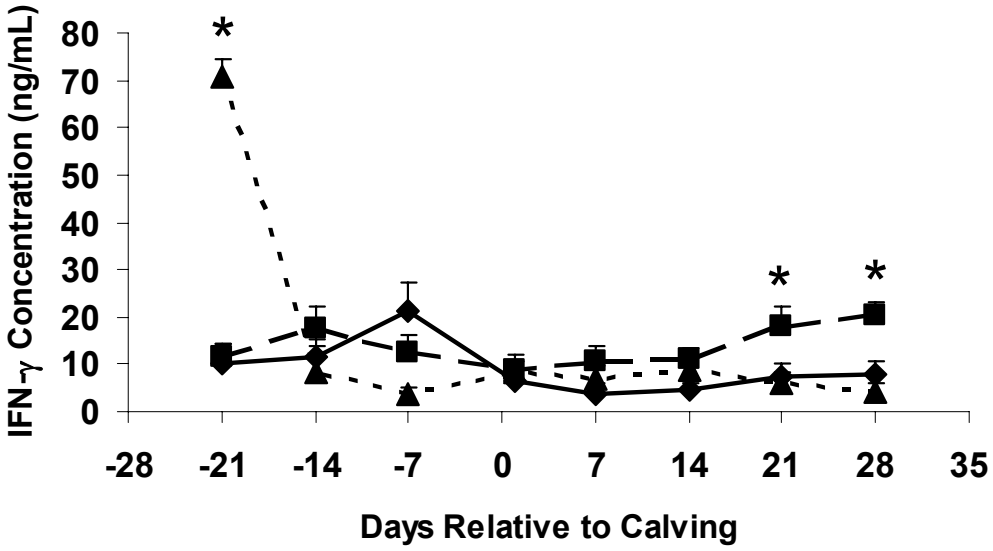

IGF-1 Mean Relative Expression (ddCt)
1.2
1
0.8
0.6
0.4
0.2
0
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving
♦ Control
■ Subclinical

Figure 4.

A.


IFN-γ Concentration (ng/mL)
80
70
60
50
40
30
20
10
0
*
*
*
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving

B.

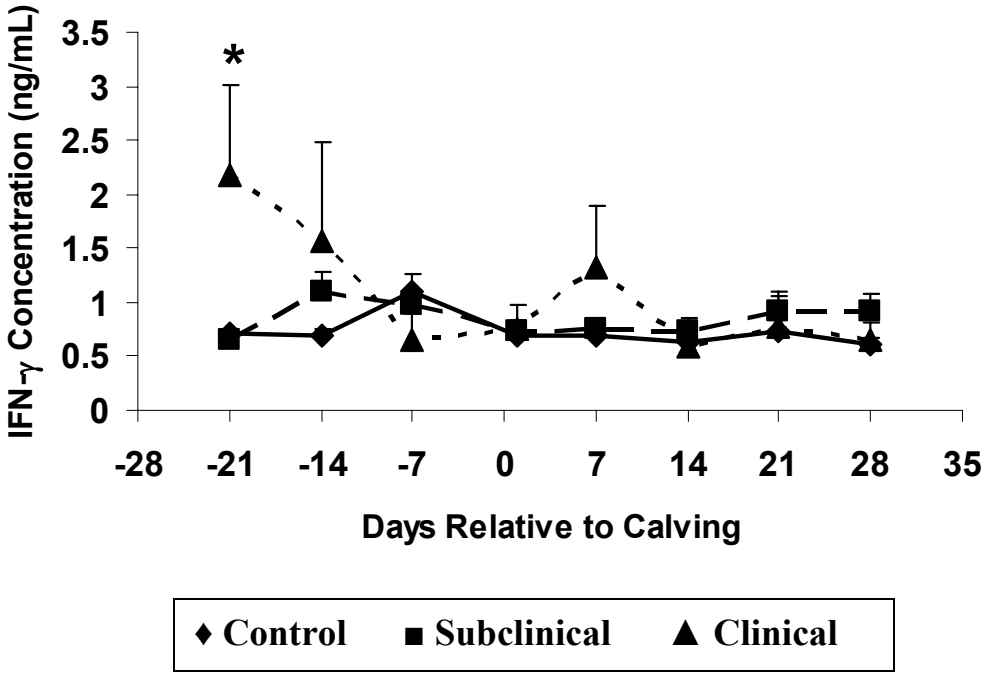

IFN-γ Concentration (ng/mL)
3.5
3
2.5
2
1.5
1
0.5
0
*
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving
♦ Control
■ Subclinical
▲ Clinical

Figure 5.

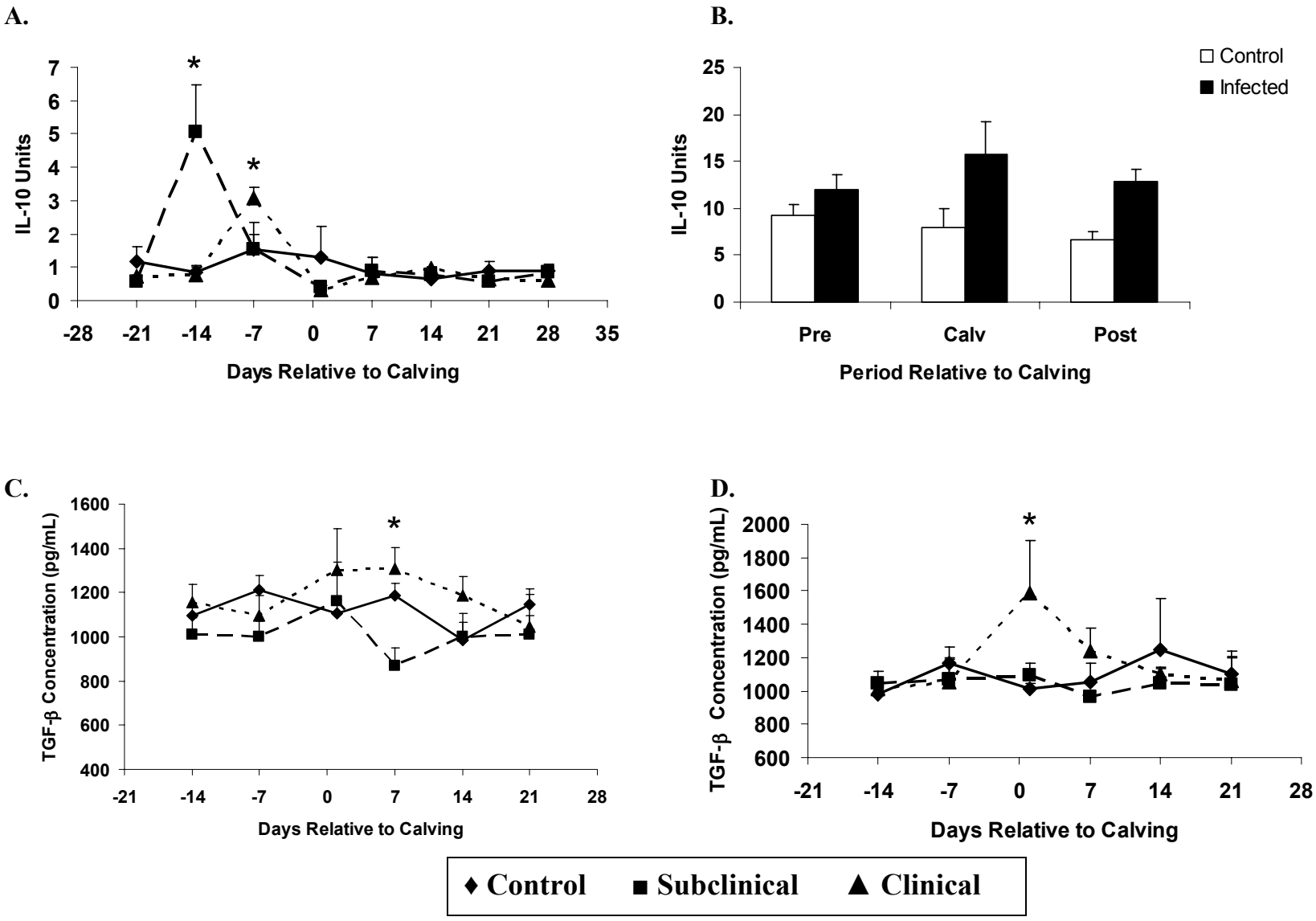
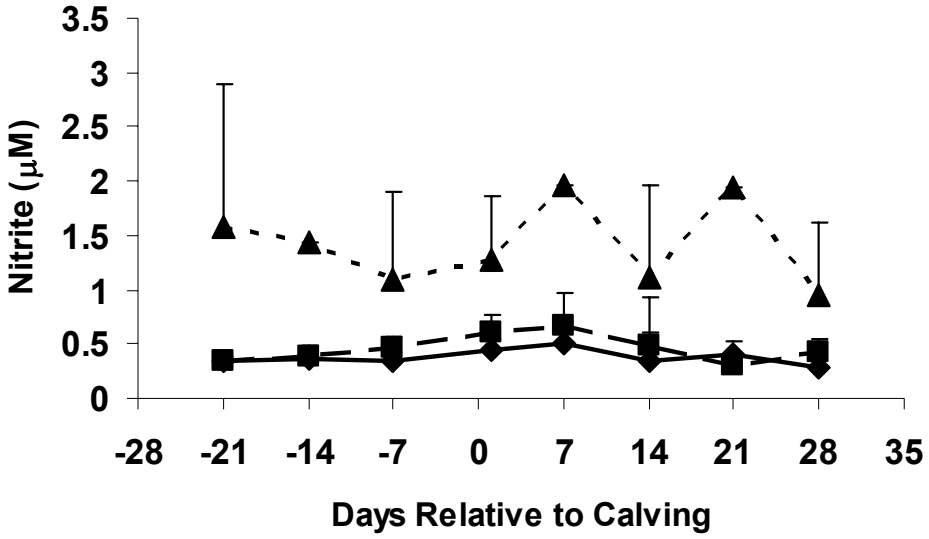

A.
IL-10 Units
Days Relative to Calving
B.
Control
Infected
IL-10 Units
Pre
Calv
Post
Period Relative to Calving
C.
TGF-β Concentration (pg/mL)
Days Relative to Calving
D.
TGF-β Concentration (pg/mL)
Days Relative to Calving
♦ Control ■ Subclinical ▲ Clinical

Figure 6.
A.


3.5
3
2.5
2
1.5
1
0.5
0
Nitrite (μM)
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving

B.

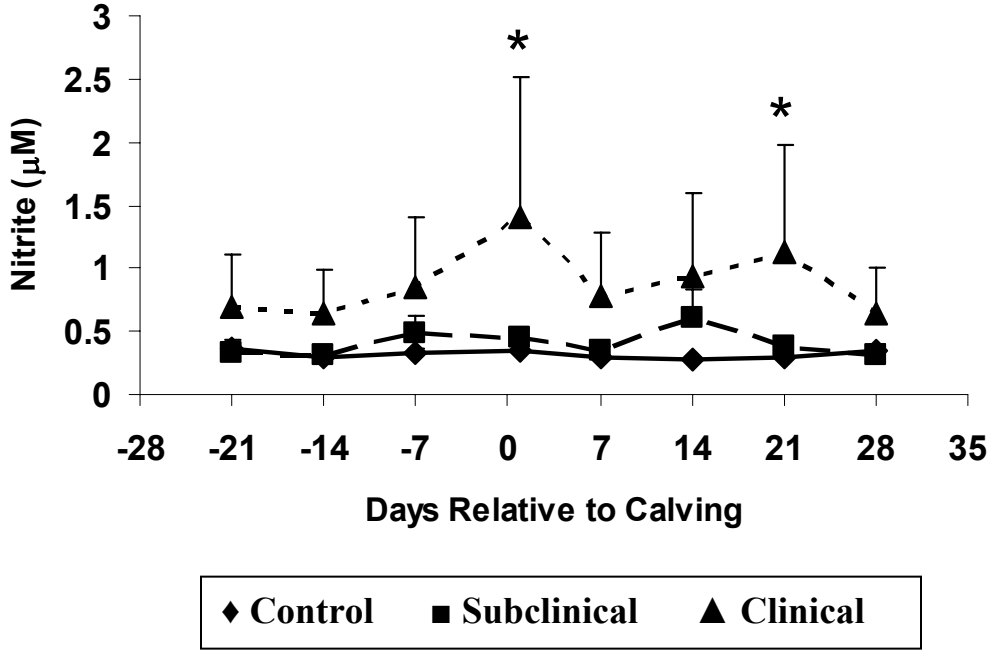
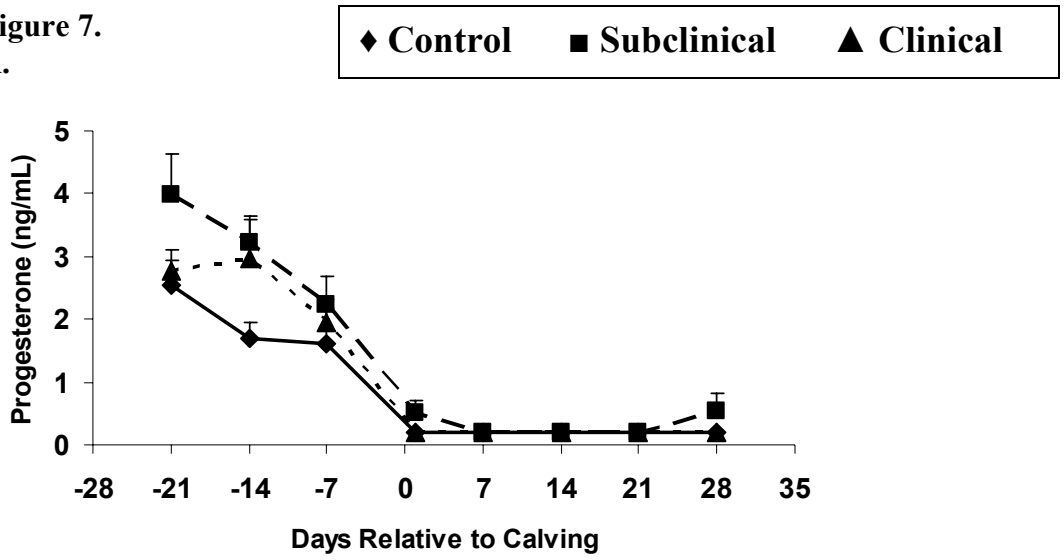
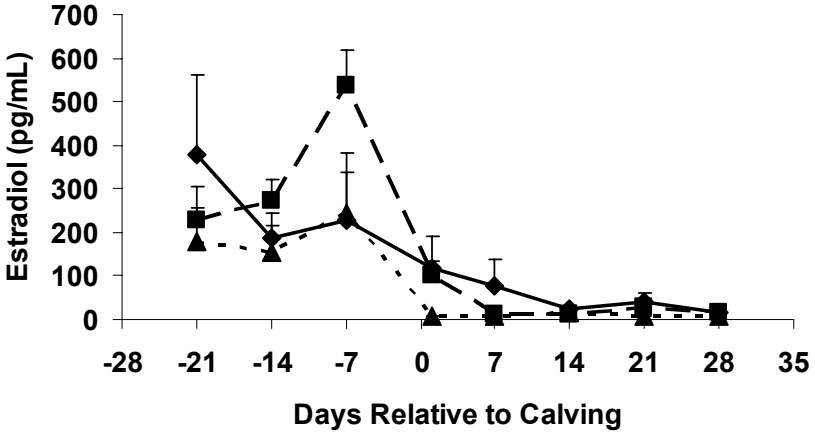

3
2.5
2
1.5
1
0.5
0
Nitrite (μM)
*
*
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving
♦ Control
■ Subclinical
▲ Clinical

Figure 7.

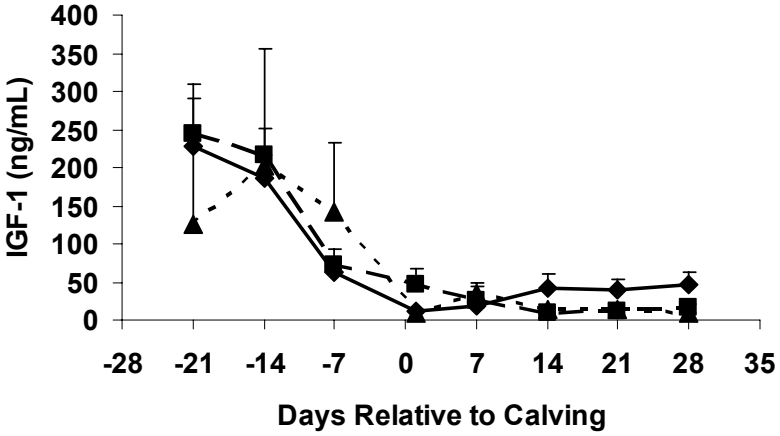
A.


♦ Control
■ Subclinical
▲ Clinical
Progesterone (ng/mL)
0
1
2
3
4
5
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving

B.


Estradiol (pg/mL)
0
100
200
300
400
500
600
700
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving

C.


IGF-1 (ng/mL)
0
50
100
150
200
250
300
350
400
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving

CHAPTER THREE

PARTURITION INVOKES CHANGES IN PERIPHERAL BLOOD MONONUCLEAR CELL POPULATIONS IN HOLSTEIN DAIRY COWS NATURALLY INFECTED WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

A paper submitted to *Veterinary Immunology and Immunopathology*

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ABSTRACT

Johne's disease is characterized by a protracted period of subclinical infection. Infected cows may remain in the subclinical state until stressors such as parturition and lactation invoke more clinical signs of disease. The objective of this study was to evaluate changes in the percentages of $CD4^+$, $CD8^+$, and γδ T-cells, B-cells, monocytes, as well as the expression of the activation marker, CD5, on these cell subpopulations in the peripheral blood of dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) during the periparturient period. Peripheral blood mononuclear cells (PBMCs) were collected from 3 wk pre- to 4 wk post-calving and sampled fresh or incubated for 7 d. Day 7 cultures were further exposed to live MAP at a 10:1 MOI (bacteria to adherent monocyte-derived macrophages) and cultures were incubated for an additional 24 h. Fluorescent antibody labeling of lymphocyte subsets and monocytes was conducted and analyzed with flow cytometry. In fresh mononuclear cells, subclinical cows expressed a greater percentage of

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$CD8^+$ and γδ T-cells compared with clinical cows. The percentage of $CD4^+$ T-cells increased in clinical cows as parturition approached. During the postpartum period, clinical cows had greater CD4:CD8 ratios compared with subclinical and control cows. Day 8 after parturition, uninfected PBMCs from clinical cows had greater percentages of $CD14^+$ cells compared with subclinical cows. When stimulated with MAP, there was no effect of infection group or parturition on percentage of cells expressed. In freshly isolated PBMCs, clinical cows expressed lower percentages of $CD4^+/CD5^{bright}$ and $CD8^+/CD5^{bright}$ compared with control cows, but greater percentages of $CD5^{dim}$ cells for all lymphocyte subsets. These results suggest changes in the percentages of lymphocyte subsets, monocytes, and CD5 markers are modulated by both infection status and the periparturient period.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*, lymphocytes, periparturient, peripheral blood mononuclear cells.

1. INTRODUCTION

Johne's disease (JD), caused by the intracellular pathogen *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is estimated to infect more than 22% of US dairy herds and cost the US dairy industry up to $250 million annually (Ott et al., 1999). In general, dairy cows will become infected with MAP as neonates through fecal-oral transmission. Once infected, cows may remain in the subclinical, or asymptomatic, stage of the disease for several years (Larsen et al., 1975). Stressors, such as parturition, may induce the transition from subclinical to clinical stage of the disease. Clinical animals are characterized by fecal shedding of the bacteria, intermittent but persistent diarrhea, progressive weight loss, and

eventual death. The exact mechanism that triggers the progression of the disease remains unknown.

Anecdotal observations suggest that dairy cows infected with MAP may demonstrate increased signs of clinical disease during the weeks following parturition. Research on what prompts the progression of disease from the asymptomatic subclinical state to a more clinical state during this time period is lacking. The transition period, defined as three weeks prior to and the three weeks following parturition, represents a time of physiological stress for the dairy cow. Metabolic changes such as the rapid increase in non-esterified fatty acid concentrations (Radcliff et al., 2003; Karcher et al., 2007), accompanied by a postpartum decline in both blood glucose (Radcliff et al., 2003) and calcium concentrations (Kimura et al., 2006), present challenges to the dairy cow. Rapid fluctuations in both serum progesterone and estradiol during the periparturient period contribute additional stressors (Weber et al., 2001; Radcliff et al., 2003). In addition, the cow is forced to deal with immunosuppression characterized by decreased lymphocyte function (Kehrli et al., 1989a; Meglia et al., 2005) and the decreased ability of neutrophils to migrate and phagocytize (Kehrli et al, 1989b; Lee and Kehrli, 1998). Parturition has a major impact on the number of T- and B-cells, both components of the adaptive immune system, and the number of monocyte/macrophages, effectors of the innate immune system in the peripheral blood of healthy dairy cows. Studies have noted dramatic decreases in the percentage of peripheral blood $CD4^+$ T-cells and γδ T-cells at parturition (Van Kampen and Mallard, 1997; Kimura et al., 1999). In contrast, increased activity of $CD8^+$ lymphocytes has been observed in cows at calving compared with mid to late lactating cows (Shafer-Weaver and Sordillo, 1997). The percentage of B-cells in peripheral blood was highest immediately prior to and lowest

immediately following parturition (Van Kampen and Mallard, 1997). In addition, the number of monocytes and monocyte-derived macrophages were increased at calving (Kimura et al., 2002). The total number and percentages of $CD4^+$ and $CD8^+$ (α/β T-cells), γδ T-cells, and B-cells, play a significant role in the ability of the animal to respond to an infection.

In paratuberculosis, the progression from a subclinical to a clinical stage of disease is characterized by a shift from cell-mediated (Th1) immunity to an antibody-mediated (Th2) humoral response. This shift in Th1 to Th2 immunity is characterized by a decreased percentage of peripheral blood T-cells and an increase in the percentage of B-cells for clinically infected cows (Waters et al., 1999; Koets et al., 2002). More specifically, the percentages of γδ and $CD4^+$ T-cells in peripheral blood are remarkably decreased in clinical cows compared with healthy controls (Koets et al., 2002). The CD4:CD8 ratio is also decreased in chronically infected animals as the number of $CD8^+$ T-cells does not seem overtly affected by the transition to a clinical disease state (Koets et al, 2002). The decline in $CD4^+$ T-cells observed in clinical cows further illustrates the compromised nature of the immune system as these cells are key effectors of Th1-mediated immunity through the secretion of IFN-γ, a cytokine that is credited for controlling mycobacterial infections (Cooper et al., 1993; Flynn et al., 1993).

To date, limited research is available characterizing detailed aspects of periparturient immunosuppression in the dairy cow. Further, it is not clear what impact the periparturient period and its associated stressors may have on host immunity in cows with paratuberculosis. Therefore, the objective of this study was to determine the percentages of $CD4^+$, $CD8^+$, and γδ T-cells, B-cells, and monocytes in the peripheral blood of dairy cows naturally infected with MAP during the periparturient period as compared with healthy control cows. In

addition, cell populations were further delineated by staining for CD5, a marker for T- and B-cell activation.

2. MATERIALS AND METHODS

2.1 Animals

Twenty-one multiparous Holstein cows and two primiparous Holstein cows (age range from 3 to 6 yr) were grouped according to infection status. These groups consisted of 1) noninfected healthy control cows (n = 5), 2) cows naturally infected with MAP, but asymptomatic (n = 14), and 3) naturally infected cows with clinical Johne's disease (n = 4). The 2 primiparous cows were both in the subclinically infected group. The stage of infection was determined by fecal shedding of MAP and IFN-γ. Infection was monitored bacteriologically for the fecal shedding of MAP by standard culture methods (Stabel, 1997). By definition, clinical animals were shedding more than 100 CFU/tube and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 CFU/tube. The noninfected control cows were characterized by repeated negative fecal cultures performed quarterly over a 3- to 5-yr period. In addition, these animals were negative on any serologic assays (i.e., production of antibody specific for MAP and IFN-γ) performed during that period. All animals were housed in American Association for Accreditation of Laboratory Animal Care-accredited facilities, and all procedures performed on animals were approved by the Institution Animal Care and Use Committee (National Animal Disease Center [NADC], Ames, Iowa).

2.2 Blood Collection and Culture Conditions

Blood was collected from the jugular vein in 2x acid-citrate-dextrose (ACD; 1:10). For each animal, blood was collected at -21, -14, -7, +1, +7, +14, +21, and +28 days relative to calving (DRTC). Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat fractions of blood. PBMCs were resuspended in complete media [RPMI-1640 (Gibco, Grand Island, NY) with 10% fetal calf serum (Atlanta Biologics, Atlanta, GA), 100 U of penicillin G sodium (Gibco, Grand Island, NY) per mL, 100 μg of streptomycin sulfate (Gibco, Grand Island, NY) per mL, 0.25 μg of amphotericin B (Gibco, Grand Island, NY) per mL, and 2 m*M* l-glutamine (Gibco, Grand Island, NY)]. Cells were cultured at 2.0 x 10^6/mL in 48-well flat-bottomed plates (Corning Incorporated, Corning, NY). Briefly, plates were centrifuged at 400 x g for 5 min and the supernatant was decanted. Cells were resuspended gently in 300 μl of PBS (137 m*M* sodium chloride, 10 m*M* phosphate, 2.7 m*M* potassium chloride; pH 7.4). In 96-well round bottom plates (Corning Incorporated, Corning, NY), 50 μL of the cell suspension was added to wells containing 50 μL of primary monoclonal antibody to $CD4^+$, $CD8^+$, γδ T-cells, B-cells, monocytes, and CD5 (Table 1). All wells received 10 μg/mL of DAPI (4'-6-diamidino-2-phenylindole; Sigma, St. Louis, MO) to differentiate live from dead cells and allow gating on viable cells. Cells then were incubated at 4°C for 30 min. After incubation, plates were centrifuged at 400 x g for 2 min at 4°C and the supernatant decanted. Secondary antibody cocktail (100 μL) consisting of fluorescein-conjugated anti-mouse IgM (Southern Biotech, Birmingham, AL), R-phycoerythrin-conjugated goat F(ab)2 anti-mouse IgG2a (Southern Biotech, Birmingham, AL), and peridinin-chlorophyll-protein complex (PerCP)-conjugated rat anti-mouse IgG1 (Becton Dickinson, San Jose, CA) diluted 1:312, 1:625, and 1:42 respectively, in PBS with 1% fetal

calf serum and 0.04% sodium azide was then added to designated wells. After incubation the plate was centrifuged again at 400 x g for 2 min at 4°C. The cells then were suspended in 200 μL of BD FacsLyse (BD Biosciences, San Jose, CA) for immediate flow cytometric analysis.

Additional cultures were incubated for 7 d in complete medium for flow cytometric analyses. Replicate wells were set up concurrently and incubated under the same conditions for enumeration of monocyte-derived macrophages in the cultures. After 7 d of incubation, nonadherent cells were removed from the plates and the wells were washed with cold 1× PBS to remove the adherent cells from the plates. The adherent cells were approximately 80 to 90% monocyte-derived macrophages as determined by staining and were quantified with a cell counter prior to the addition of live MAP. *M. avium* subsp. *paratuberculosis* strain K-10 (NADC) was added at a ratio of 10 bacteria per adherent PBMC to the experimental wells (Khalifeh and Stabel, 2004). Control wells (medium alone; 8 d control PBMC) and infected wells (MAP infected; 8 d infected PBMC) were then incubated for an additional 24 h. Cells then were prepared for flow cytometric analysis by using the same procedure as described above.

2.3 Bacteria

M. avium subsp. *paratuberculosis* strain K-10 (NADC) was prepared as previously described (Khalifeh and Stabel, 2004). Bacteria were grown in Middlebrook 7H9 broth (pH 6.0) supplemented with mycobactin J (2 mg/L; Allied Monitor, Fayette, MO) and oleic acid-albumin-dextrose complex (Becton Dickinson Microbiology, San Jose, CA). The bacteria were harvested, washed 3 x with PBS (pH 7.4; 0.15 *M*), and resuspended in PBS to a final

concentration of 10^9/mL as determined by the absorbance at 540 nm. Bacterial stocks then were frozen in PBS at -80°C until used in the experiments. Prior to in vitro infection, frozen bacterial stocks were thawed, and clumps were dispersed by brief sonication at 25 W for 40 s with a Tekmar sonic disturber (Tekmar, Lorton, VA). The viable cells in the frozen bacterial stocks were determined by culturing on HEYM. Viable cells in stocks typically were decreased after thawing and sonication to approximately 10^8 CFU/mL.

2.4 Flow Cytometric Analysis

Samples were evaluated by analyzing 30,000 events per sample using a FACScan flow cytometer (Cell Quest Software; Becton Dickinson, San Jose, CA). Analysis was conducted by gating on mononuclear cells based on forward and side scatter characteristics (FlowJo, Tree Star, Inc., Ashland, OR). The percentage of $CD4^+$, $CD8^+$, γδ T-cells, B-cells, and monocyte/macrophage populations were determined from the gated mononuclear cell population. Percentages of CD5 positive cells within these cell populations also were determined.

2.5 Statistical Analysis

Percentages of each cell population were analyzed by using the PROC Mixed analysis of SAS (PROC MIXED in SAS® PC Windows Version 9.1.3 software). For repeated measurements, the model included the fixed effects of infection group and DRTC, random effects of cow within infection group by time, the interactions of fixed effects, and the residual error. The covariance structure utilized was compound symmetry in all analyzed parameters. Significant differences because of treatment group were considered different if

$P < 0.05$ and considered a tendency towards significance if $0.05 \leq P \leq 0.15$. Values were reported as least square means ± standard errors of the mean unless noted otherwise.

3. RESULTS

3.1 Mononuclear cell populations in freshly isolated cells

An overall effect of parturition ($P < 0.05$) caused a significant decline in the percentage of total mononuclear cells from -7 to +1 DRTC for all animals regardless of infection status (data not shown). Values returned to pre-calving values by +14 d. On +7 d and +21 d after calving, clinical and subclinical cows had decreased ($P < 0.05$) numbers of mononuclear cells compared with the control cows. A significant ($P < 0.05$) DRTC effect was observed throughout the periparturient period for the percentage of $CD4^+$ cells in fresh PBMC cultures (Fig. 1A). Compared with the prepartum period, the percentage of $CD4^+$ T-cells increased at parturition for clinically infected cows ($P < 0.08$), whereas healthy control cows showed a gradual decline in number of $CD4^+$ T-cells from -21 d to -7 d. A highly significant ($P < 0.01$) infection group x parturition effect was observed throughout the study. At +7 d and +21 d, infected cows had a greater ($P < 0.01$) percentage of $CD4^+$ T-cells in total PBMC compared with the control cows. There was an overall effect of infection group on the percentage of $CD8^+$ T-cells in the fresh PBMC cultures (Fig. 1B). On average, the percentage of $CD8^+$ T-cells was greater ($P < 0.05$) in subclinically infected cows (20.3% ± 2.1) compared with clinically infected (10.3% ± 3.77) and control (10.3% ± 3.3) cows. An interaction of infection group and DRTC was observed ($P < 0.01$) as subclinical cows had greater percentages of $CD8^+$ T-cells compared with the other groups on all sampling days during the postpartum period.

The CD4:CD8 ratio was dissimilar within the infected groups with higher ($P < 0.01$) CD4:CD8 ratios noted for clinical compared to subclinical cows (Table 2). There was a significant ($P < 0.05$) infection group x DRTC interaction with higher CD4:CD8 ratio observed on d +1, +7, and +14 of the study.

In fresh PBMC cultures, the overall percent of γδ-TCR$^+$ cells was lower ($P < 0.01$) in clinical cows (1.7% ± 0.9) compared with subclinical cows (4.4% ± 0.5), with a trend ($P < 0.06$) to be lower than control cows (3.9% ± 0.8) (Fig. 1C). There was no significant effect at parturition; however, both control and subclinical cows tended to have increased ($P < 0.12$) numbers of γδ T-cells as parturition approached, with further increases noted for subclinically infected cows in the postpartum period.

There was no effect of infection group or parturition on the percentage of B-cells in this study (Fig. 2A). However, the percentage of B-cells from subclinical cows declined as parturition approached ($P < 0.03$). An increase in the percentage of B-cells from clinical cows was noted from -14 d to +7 d ($P < 0.06$). Similarly, there was no effect of infection or parturition on the percentage of CD14$^+$ cells in fresh PBMCs (Fig. 2B), but a trend for an infection group x DRTC effect ($P < 0.12$) was observed. Subclinical cows expressed a greater percentage of CD14$^+$ cells on +7, +14, and +21 d compared with control and clinical cows.

3.2 Effects of in vitro infection with M. avium subsp. paratuberculosis on mononuclear cell populations in 8 d cultures

PBMCs from clinical cows incubated with medium alone had an overall lower ($P < 0.05$) percentage of mononuclear cells (61.3% ± 3.2) compared with both control (71.2% ±

2.8) and subclinically infected cows (68.5% ± 1.6) (data not shown). Statistically significant differences due to infection status were only observed during the prepartum period (infection group x DRTC effect, $P < 0.01$). For control and subclinical cows, the percentage of mononuclear cells declined from -7 to +1 d and rapidly increased by +7 d (data not shown). Similar trends were observed after in vitro infection with MAP, with lower percentages of mononuclear cells noted for clinically infected cows during the prepartum period compared with control and subclinical cows (infection group x DRTC effect, $P < 0.10$). A significant ($P < 0.05$) overall DRTC effect was observed for clinical cows as the number of mononuclear cells increased from -7 to +7 d and gradually decreased again by +28 d. Although not statistically significant, the control and subclinical cows had a decline in mononuclear cells from -14 to +1 d (Fig. 3). There was no overall effect of infection group on the percentage of $CD4^+$ T-cells from 8 d control PBMCs (Fig. 4A). A trend for a DRTC effect ($P < 0.09$) was noted with both control and clinical cows demonstrating a decline in $CD4^+$ T-cells as parturition approached. By +21 d, the percent had returned to prepartum values. Subclinical cows were unique in that they showed a 32% increase in $CD4^+$ T-cells beginning at -14 d and continuing until +7 d (Fig. 4A). By +28 d, the percentage had returned to -14 d prepartum values. For 8 d infected PBMC cultures, there was an infection group x DRTC effect ($P < 0.01$) (Fig. 4B). For subclinical cows, the percentage of $CD4^+$ T-cells increased from 21.2% ± 2.1 at -14 d to 35.6% ± 2.2 at +1 d ($P < 0.01$), followed by a decline during the postpartum period. Interestingly, in contrast to subclinically infected cows, in clinically infected cows the percentage of $CD4^+$ T-cells decreased from 33.4% ± 5.8 at -21 d to 18.0% ± 2.2 at +1 d, rebounding to 28.1% ± 4.6 by +21 d. The percentage of

$CD4^+$ T-cells from control animals remained relatively constant during the periparturient period until an increase was noted at +14 d.

There was no overall effect of infection group on the percent of $CD8^+$ T-cells from 8 d control PBMC; however, an increase in $CD8^+$ T-cells was observed at parturition for all cows (Fig. 5). Interestingly, clinical cows had a brief increase ($P < 0.01$) in $CD8^+$ T-cells from 15.7% ± 0.7 at +7 d to 31.6% ±5.1 at +14 d. No effect of parturition or infection group was observed on the $CD8^+$ population in 8 d infected PBMCs (data not shown). There was no effect of infection group on the CD4:CD8 ratio in either control or infected 8 d PBMC; however, a significant decrease ($P < 0.01$) in CD4:CD8 ratio was observed for control and clinically infected cows as parturition approached (data not shown).

There was no overall effect of infection group on percent of γδ-TCR^+ or B-cells from control or 8 d infected PBMCs (data not shown). However, a significant ($P < 0.04$) DRTC effect was noted on +1 d with clinical cows having higher numbers of B-cells in 8 d control PBMC (70.8% ± 7.6) compared with subclinical (56.7% ± 4.7) and control (45.8% ± 4.6). Clinical cows had a greater ($P < 0.05$) percentage of $CD14^+$ cells in 8 d control PBMC (39.1% ±2.7) compared with subclinical cows (31.5% ±1.4), with a tendency for higher numbers of monocyte-derived macrophages compared with the control cows ($P < 0.10$). Parturition did not affect the percentage of $CD14^+$ cells in 8 d control PBMC (Fig. 6). There was no overall effect of infection group or parturition on $CD14^+$ cells from 8 d infected PBMCs (data not shown).

3.3 CD5 expression on mononuclear cell populations after fresh isolation or 8 d of culture

Overall, in fresh PBMCs, clinical cows expressed lower ($P < 0.01$) percentages of $CD4^{+}/CD5^{bright}$ T-cells and $CD8^{+}/CD5^{bright}$ T-cells compared with the control cows and tended to have lower expression of these subpopulations than the subclinical cows ($P < 0.10$) (Fig. 7A/B). There was no overall effect of periparturient period. However, there was a significant increase ($P < 0.01$) in expression of $CD4^{+}/CD5^{bright}$ T-cells for control cows between -14 d (46.82%) and the day of calving (79.2%). This same pattern was observed for $CD8^{+}/CD5^{bright}$ T-cells. Control cows also expressed a higher ($P < 0.05$) percentage of $\gamma\delta TCR^{+}/CD5^{bright}$ T-cells in fresh PBMC compared with the subclinically infected cows (Fig. 7C). Although parturition did not affect the percentage of $\gamma\delta TCR^{+}/CD5^{bright}$ T-cells, infected cows did have decreased expression in the postpartum period from +1 d through +21 d. Parturition did not affect the percentage of B-cell/$CD5^{bright}$ cells, but there was a strong trend towards lower expression for both subclinical ($P < 0.11$) and clinical ($P < 0.13$) cows compared with control cows (Fig. 7D).

In contrast, clinical cows expressed a greater percentage of $CD4^{+}$ cells/$CD5^{dim}$ cells compared with control (21.6% ± 4.8 vs. 40.44% ± 5.5; $P < 0.05$). There was no overall effect of parturition, but the percentage of cells from subclinical cows increased from -7 d to +21 d ($P < 0.05$) and decreased in control cows from -14 d to +1 ($P < 0.05$) (Fig. 8A). The percentage of $CD8^{+}$ cells/$CD5^{dim}$ cells was greater in clinical cows ($P < 0.01$) and tended to be greater in subclinical cows ($P < 0.06$) compared with the control cows (Fig. 8B). For clinical cows, the percentage rapidly increased from -7 d to +14 d ($P < 0.01$). During the postpartum period, subclinical and clinical cows expressed a greater percentage of $\gamma\delta$ cells/$CD5^{dim}$ compared with the control cows ($P < 0.05$) (Fig. 8C). Overall, clinical cows

expressed a greater percentage of B-cell/CD5^{dim} cells compared with the control cows ($P < 0.05$) (Fig. 8D).

There was no overall effect of infection or parturition on CD4^{+}/CD5^{bright} T-cells in 8d control (Table 3) or infected (Table 4) PBMCs, although there was an infection group x DRTC effect ($P < 0.09$). There was an overall effect of parturition ($P < 0.07$) (Table 3) with overall percentages of CD8^{+}/CD5^{bright} T-cells decreasing from -21 d to +28 d ($P < 0.05$). In 8 d infected PBMCs, there was an overall DRTC effect ($P < 0.01$) and an infection group x DRTC effect ($P < 0.01$). In the control cows, the percentage of γδ/CD5^{bright} T-cells cells declined from 39.8% ± 5.8 at -21 d to 9.2% ± 2.9 by +7 d ($P < 0.01$). The percentage then rapidly increased to +21 d. A DRTC effect was observed in 8 d infected PBMC as the percentage of B-cell/CD5^{bright} cells increased in the prepartum period from -14 d to +1 d ($P < 0.01$) for subclinically infected cows, whereas an increase in B-cell/CD5^{bright} cells was also observed from +7d to +21 d postpartum in control cows ($P < 0.05$).

4. DISCUSSION

Infections caused by MAP have an overwhelming impact on the health of the dairy cow and ultimately on the dairy industry. Limited research has focused on changes in populations of peripheral blood mononuclear cells in dairy cows naturally infected with MAP during the periparturient period. The populations of B and T lymphocytes subsets in the peripheral blood have a direct impact on the ability of the animal to respond to new and existing infections. There are two distinct lineages of T-cells based upon the T-cell receptor, αβ TCR and γδ TCR, with commitment to a lineage occurring early in T-cell development (MacDonald et al., 2001). T-cells committing to the α:β TCR lineage will further develop

into either a $CD4^+$ or $CD8^+$ phenotype. $CD4^+$ T-cells, also known as T-helper cells, play an active role in initiating both the humoral and cell-mediated immune responses. In mycobacterial infections, $CD4^+$ T-cells are recognized as the primary producer of IFN-γ, a key proinflammatory cytokine required for activation of macrophages and subsequent clearance of infection (Stuehr and Marletta, 1987; Flynn et al., 1993).

The percentage of $CD4^+$ T-cells in PBMCs from healthy adult dairy cattle is approximately 25-35%. Previous studies offer contrasting results for the effects of parturition on the number of circulating $CD4^+$ T-cells. Studies with dairy cows have supported an increase (Van Kampen et al., 1999), decrease (Kimura et al., 1999), or no change (Harp et al., 2004) in the percentage of $CD4^+$ T-cells at parturition. In the current study, the number of $CD4^+$ T-cells in the peripheral blood averaged 21.6% ± 2.1, 24.3% ± 1.3, and 25.7% ± 2.4 for control, subclinical, and clinical cows, respectively, with no differences noted between the infection groups. No effect of MAP infection on percentages of $CD4^+$ T-cells is in agreement with a previous study that did not observe differences in $CD4^+$ T-cell percentages in fresh PBMCs isolated from healthy and MAP-infected periparturient cows (Harp et al., 2004). However, these results are in contrast to one study that reported a decrease in $CD4^+$ T-cells in peripheral blood and the ileum of clinically infected cows compared with their subclinically infected counterparts (Koets et al., 2002). A loss of $CD4^+$ T-cells likely contributes to the progression of granulomatous enteritis noted in the clinical stage of disease. Interestingly, stimulation of 8 d PBMCs with MAP resulted in an increase in the percentage of $CD4^+$ T-cells during the prepartum period for subclinical cows with a decrease noted for clinical cows. These data would suggest that $CD4^+$ T-cells from subclinically infected cows are able to proliferate in response to antigen during periods

of physiologic stress, whereas clinically infected cows may be compromised in their ability to respond.

$CD8^+$, or cytotoxic T-cells are important for their ability to recognize viral and bacterial antigens and to target T-cells displaying these antigens for apoptosis (Kagi et al., 1994). Similar to $CD4^+$ T-cells, $CD8^+$ T-cells also secrete pro-inflammatory cytokines, IFN-γ and TNF-α, making them equally important in controlling infection (Fong and Mosmann, 1990; Berg et al., 2002). Studies depleting mice of their $CD8^+$ T-cell populations by using anti-CD8 antibodies have demonstrated a protective role for $CD8^+$ cells in *M. tuberculosis* (Muller et al., 1987). In the current study, the percentage of $CD8^+$ T-cells ranged from 10-22% which is equivalent to published values for adult dairy cows (Harp et al., 1991; Meglia et al., 2005). An interactive effect of infection group and day relative to calving was observed with subclinically infected cows expressing 2-fold higher percentages of $CD8^+$ cells in the postpartum period. Similar studies with healthy cows have reported a decline in $CD8^+$ T-cell percentages at parturition (Kimura et al., 1999; VanKampen et al., 1999). This is in contrast to two studies reported by Harp et al. (1991 and 2004) that suggest that $CD8^+$ T-cells in peripheral blood are not influenced by parturition. In Harp's study, the percentage of $CD8^+$ T-cells was 9% at calving and 12% at +21d. In contrast to our data, other groups also have reported no differences in percentages of $CD8^+$ T-cells between healthy, subclinical, and clinical cows (Koets et al., 2002). However, these observations were not made during the periparturient period. Cytotoxic killing of $CD4^+$ cells by γδ T cells has been reported in infections caused by MAP (Chiodini and Davis, 1992). However, the ability of γδ T cells to exert this effect is limited when $CD8^+$ cells are present because of the inactivation of this group by a subset of $CD8^+$ T-cells (Chiodini and Davis, 1993). In our study, the increase in

both $CD8^+$ and γδ T cells observed during the postpartum period may be an example of the dynamic regulation that exists among the T-cell subsets. Another explanation for the increase in $CD8^+$ T-cells in the subclinically infected animals may be that this T cell subpopulation is highly activated as a result of the cytokine microenvironment associated with the initial stages of MAP-infection and that this response provides an early defense system against the invading bacteria (Lertmemongkolchai et al., 2001; Yajima et al., 2002).

The CD4 to CD8 T-cell ratio is often used as an indicator of immune status. For example, in HIV-infected populations, a diminished CD4:CD8 ratio may be used to accurately predict the occurrence of an AIDS-related complication (Pirzada et al., 2006). In advanced mycobacterial infections, there is a reduction in the number of $CD4^+$ T-cells that is associated closely with a loss of Th1-mediated immune function. A decrease in the number of $CD4^+$ T-cells, resulting in diminished CD4:CD8 ratio is observed in cases of pulmonary tuberculosis (Pilheu et al., 1997; Uppal et al., 2004). One mechanism that may account for the fewer CD4+ T cells is an increase in cellular apoptosis such as was observed in BALB/c mice infected intraperitoneally with *M. tuberculosis* (Das et al., 1999). When compared with subclinical cows, clinical cows have diminished CD4:CD8 ratios in the peripheral blood, with little variation in the percentage of $CD8^+$ T-cells (Chiodini and Davis, 1992; Koets et al., 2002). On the first sampling day of the current study (d -21), the CD4 to CD8 ratios were significantly lower for infected cows compared with controls (1.9 vs 4.0). This follows the aforementioned paradigm for MAP infection. However, the periparturient period triggered differences in the peripheral blood CD4 to CD8 T-cell ratios between cows in different stages of infection. Subclinically infected cows had lesser CD4 to CD8 ratios, whereas the clinically infected cows had higher CD4:CD8 ratios during the immediate postpartum period. The

CD4:CD8 ratio does not seem to be influenced by parturition in healthy dairy cows, with ratios averaging 3:1 (Van Kampen and Mallard 1997; Kimura et al., 1999). However, during the first 90 d in lactation, the ratio has been reported to decline to 1.81 (Park et al., 1992). Values in our study averaged 2.7 ± 0.6, 1.9 ± 0.4, and 3.9 ± 0.7 for control, subclinical, and clinical cows, respectively, throughout the periparturient period. These results suggest there was an interaction between the effects of MAP infection and the dynamic events occurring during the periparturient period. Although a decrease in the CD4 to CD8 ratio is indicative of immunosupression, recent information on the role of CD8 cells in the host response to mycobacterial infections suggests this population may provide additional protection by augmenting the innate immune response through secretion of IFN-γ (Berg et al., 2002).

γδ T-cells seem to initiate immune responses and may regulate host inflammatory response to infection although their specific roles in host immunity are still relatively undefined. The percentage of γδ T-cells is greatest in the calf (40%) and gradually declines to approximately 5% of adult PBMCs (Hein and MacKay, 1991). In fresh PBMCs, clinically infected cows had a much lower percentage of γδ T-cells compared with subclinically infected and control cows. This response is in agreement with a study reporting that the population of γδ T-cells from freshly isolated PBMCs was much greater in control cows (21.8%) compared with cows clinically infected with MAP (7.3%) (Koets et al., 2002). In the current study, an increase in γδ T-cells was noted as parturition approached, with further increases noted throughout the postpartum period for subclinically infected cows. Typical observations for dairy cows during the periparturient period include either declining numbers of γδ T-cells (Van Kampen and Mallard, 1997; Kimura et al., 1999) or no change in the

percentage of γδ T-cells (Park et al., 1992; Harp et al., 2004; Meglia et al., 2005). Kimura et al. (1999) reported a decline in γδ T-cells from 7.3% on -13 d to 5.4% on +5 d. These values are similar to values obtained for control and subclinically infected cows in our study, but are higher than the average values in this study for clinically infected cows (1.66%). The exact function of γδ T cells remains to be found, although there is evidence to suggest that these cells play a significant role in the innate immune response to initial mycobacterial infection. Early infection with *M. tuberculosis* results in increased numbers of activated γδ T cells that are capable of producing the proinflammatory cytokine, IFN-γ (Tsukagucki et al., 1995). Furthermore, stimulation of bovine γδ T cells with mycobacterial products resulted in expansion and production of IFN- γ (Vesosky et al., 2004). The lower percentage of γδ T cells observed in the clinically infected cows in our study may be an attempt to limit the severe inflammation and tissue damage caused by an abundance of proinflammatory cytokines in the subclinical stage of Johne's disease.

In the current study, the percentage of B-cells across infection groups ranged from 28-35%. Neither infection status of the cows nor parturition had an effect on the overall percentages of B-cells. Similar to the T lymphocytes, the literature gives a contrasting view of B-cell percentages in healthy cows over the periparturient period. Some studies indicate that the percentage of B-cells in peripheral blood throughout the periparturient period remains constant at 25% (Park et al., 1992; Harp et al., 2004) while others indicate increasing (Meglia et al., 2005) and decreasing (Van Kampen and Mallard, 1997) percentages during the postpartum period. In the advanced stages of Johne's disease, antibody production by B-cells does little to protect the host from the progressive MAP-infection. Waters et al. (1999) reported that population percentages of B-cells in peripheral blood were similar between

subclinically infected and control cows but were greater in clinically infected cows. This same study concluded that B cells from clinically infected, but not subclinically infected, Johne's cows fail to proliferate in response to antigen stimulation and that antigen response in the peripheral blood of these cows is absent. In our study, the increase in percentage of B cells from -14 d to +7 d in freshly isolated PBMCs for clinically infected cows is an indication of activated humoral response around the time of calving. In our study, clinically infected cows had a lower percentage of B cells prepartum and at calving compared with subclinical and control cows. The fact that this value returned to a normal level by +7 d may be an indication of activated humoral response in clinically infected cows during the postpartum period.

CD5 is expressed on all bovine T-cells and a subset of B-cells (Delpelchin et al., 1989; Naessens and Williams, 1992). In T-cells, CD5 is a regulating component of TCR-signal transduction during the double-positive stage of lineage commitment (Tarakhovsky et al., 1995). In healthy dairy cows, a subset of B-cells is $CD5^{+}$ and is associated with the B-cell receptor (Cantor et al., 2001). We have identified previously two subpopulations of $CD5^{+}$ cells ($CD5^{bright}$ and $CD5^{dim}$) in the bovine that are modified by paratuberculosis infection. Both CD5 bright and dim populations were examined as a percentage of marker positive lymphocyte subsets. For $CD4^{+}$, $CD8^{+}$, and B-cell subsets, clinical cows expressed the lowest percentage of $CD5^{bright}$ cells and the greatest percentage of $CD5^{dim}$ cells compared with the control cows. It is significant that clinical cows expressed a greater percentage of $CD5^{dim}$ B-cells (21.8% ± 3.6 in control cows vs. 33.4% ± 4.2 in clinical cows) because these cells are capable of producing the Th2 cytokine, IL-10 (Gieni et al., 1997). It has been identified that the IL-10 produced by $CD5^{+}$ B-cells acts directly on macrophages to decrease

IL-12 secretion (Sun et al., 2005). The transition from the subclinical to the clinical stage of Johne's disease is characterized by a switch from Th1- to Th2–mediated immune responses. The ability of MAP to modulate the expression of IL-10 is critical in the pathogenesis of Johne's disease. Interleukin-10 is capable of down-regulating macrophage and natural killer cell function, lowering the overall ability of T cells to proliferate, and suppressing cell-mediated immunity. Previous work has shown that proliferation of $CD5^{+}$ B-cells is inhibited by IL-12 and enhanced by IL-5, another Th2 cytokine (Vogel et al., 1996). On the basis of our data, we hypothesize that the increased percentages of $CD5^{+dim}$ B-cells by clinical Johne's cows directly inhibits cell-mediated immune responses by altering the cytokine microenvironment in favor of a Th2 response.

The results from this study contribute to the limited available information that focuses on the impact periparturient immunosuppression may have on the ability of the host to respond to progressing MAP infection. Stress induced by parturition has a major impact on the number of T- and B-cells in the peripheral blood. Our data clearly supports an interaction of parturition and infection status on these lymphocyte subsets. This interaction is best illustrated by the upregulation of $CD4^{+}$ in clinically infected cows and an increase in $CD8^{+}$ in subclinically infected cows at parturition. In these animals, these lymphocyte subsets are responding to the bacteria in an attempt to contain and limit the MAP-infection. Altering changes in lymphocyte immune responses may be one mechanism to assist the infected dairy cow in attempting to manage the highly stressful periparturient period.

CONCLUSIONS

Results of this study indicate that in dairy cows the percentages of both lymphocyte subsets and mononuclear cells are modulated by natural infection with MAP and by the periparturient period. In addition, these factors are capable of influencing expression of the activation marker, CD5, on T cell subpopulations and B cells. The data presented are important because they highlight changes in the immune response of infected cattle at parturition that may be an attempt to limit the progression of Johne's disease during the highly stressful time of parturition.

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Table 1. Primary antibodies[1]

Antigen	MAb clone	Isotype	Working MAb Concentration[2] (μg/mL)	Specificity
CD4	GC50A1	IgM	14	T-Helper cell
CD5	B29a	IgG_{2a}	7	Activation Marker
CD8	BAQ111A	IgM	14	T-cytotoxic/ suppressor cell
CD14	CAM36A	IgG_1	14	Monocytes/ Macrophages
N12	CACT61A	IgM	14	γδ-cell receptor
B lymphocyte	BAQ155A	IgG_1	7	Total B cell

[1] VMRD Inc. (Pullman, WA)
[2] Diluted in PBS with 1% fetal calf serum and 0.04% sodium azide

Table 2. CD4:CD8 ratios across the periparturient period for freshly cultured peripheral blood mononuclear cells isolated from healthy cows, subclinical, and clinical cows naturally infected with MAP.

	Days Relative to Calving							
Infection Status	-21	-14	-7	+1	+7	+14	+21	+28
Control	4.03±0.7[a]	3.32±1.1	2.41±1.0	2.03±0.6[a]	2.58±1.0[a]	2.21±0.2[a]	1.11±0.6	2.34±0.4
Subclinical	1.93±0.4[b]	2.18±0.4	2.42±0.5	1.78±0.4[a]	1.61±0.6[a]	1.22±0.2[a]	1.52±0.4	1.45±0.4
Clinical	1.97±1.8[ab]	3.43±1.4	3.20±1.3	6.08±1.3[b]	4.78±1.5[b]	5.49±0.1[b]	2.98±0.9	2.52±1.1

Subscripts indicate differences between infection groups within a given time point ($P < 0.05$).
Infection group, P <0.03
DRTC, P <0.11
Infection group X DRTC, P <0.05

Table 3. Percentage of $CD5^{bright}$ cells in CD4, CD8, γδ T-cell, and B-cell populations after 8 days in culture. Cells were PBMCs isolated from healthy, subclinical, and clinical dairy cows naturally infected with MAP.

	Infection Status	-21	-14	-7	+1	+7	+14	+21	+28
$CD4^+$	Control	53.03±10.4	45.82±10.9	60.76±8.8[a]	39.84±7.6	40.00±9.1	31.30±7.0	47.60±3.1	24.52±5.3
	Subclinical	34.07±6.3	38.76±6.0	35.23±6.7[b]	40.52±6.0	47.63±9.3	36.88±8.4	47.78±6.7	41.98±5.7
	Clinical	57.45±33.8	47.70±17.1	33.30±12.4[ab]	50.50±16.39	39.90±10.4	29.40±0.6	58.40±17.1	33.60±22.1
$CD8^+$[1]	Control	34.11±15.5	29.16±7.9	45.52±10.1	41.02±12.4	29.10±10.3	27..66±7.2	40.82±6.9	26.12±5.6
	Subclinical	32.00±5.3	30.06±6.2	31.52±5.3	31.32±6.6	42.37±9.9	21.89±4.9	36.13±7.4	21.63±4.7
	Clinical	49.96±19.6	34.37±8.7	47.73±14.8	55.70±16.1	32.63±10.4	15.90±0.4	27.03±7.1	16.65±4.2
γδ TCR_2	Control	33.78±10.1	47.20±10.9	56.82±12.5[a]	21.97±5.6[ab]	33.05±8.1	46.78±9.9[a]	52.60±11.9[a]	37.03±1.4
	Subclinical	16.77±3.7	27.24±6.8	23.74±6.1[b]	12.69±3.2[a]	13.98±3.1	14.24±3.4[b]	22.71±5.7[b]	15.39±2.2
	Clinical	27.23±15.5	27.55±8.9	30.94±9.3[b]	35.77±12.7[b]	23.03±6.8	16.74±11.8[ab]	21.45±9.3[b]	23.30±7.0
B-$cells_4$	Control	39.20±10.4	25.30±3.6	49.80±6.9[a]	37.82±5.0	37.20±7.9	33.98±8.9	33.57±7.8	24.64±4.8
	Subclinical	28.01±4.2	24.16±5.4	24.64±4.0[b]	29.38±3.8	40.11±6.3	30.90±5.9	36.64±8.6	31.33±7.0
	Clinical	54.15±21.6	32.88±12.9	37.38±13.9[b]	16.17±7.3	28.21±11.8	17.20±1.8	37.30±10.2	27.50±8.0

Superscripts indicate differences between infection groups within a given time point ($P < 0.05$).

[1] Day relative to calving effect: $P < 0.07$

[2] Infection group: $P < 0.01$

Table 4. Percentage of $CD5^{dim}$ cells in CD4, CD8, γδ T-cell, and B-cell populations after 8 days in culture. Cells were PBMCs isolated from healthy, subclinical, and clinical dairy cows naturally infected with MAP.

Cell Type	Infection Status	-21	-14	-7	+1	+7	+14	+21	+28
$CD4^+$ [1]	Control	32.30±9.7[ab]	18.22±7.6	24.70±7.4	30.12±13.4	16.12±5.7[a]	23.91±8.3[ab]	41.46±10.7	23.40±9.2
	Subclinical	16.42±4.2[a]	29.77±4.9	30.60±5.8	40.85±8.2	46.3±6.0[b]	42.03±6.5[a]	42.06±5.9	39.34±4.8
	Clinical	54.00±21.0[b]	34.0±10.5	28.38±11.5	21.31±6.1	34.10±6.9[ab]	12.45±4.9[b]	39.53±16.5	31.78±26.3
$CD8^+$ [2]	Control	8.96±0.4[a]	10.63±2.2	15.68±4.9	13.44±3.6[a]	17.17±5.5[a]	27.47±11.4	44.92±9.1	16.57±3.7
	Subclinical	15.70±1.8[a]	22.22±4.9	20.59±4.3	30.66±7.1[ab]	28.89±6.0[ab]	34.74±6.4	34.88±6.3	34.48±1.0
	Clinical	32.23±4.8[b]	24.92±8.2	20.48±4.9	25.47±3.1[b]	43.33±13.2[b]	17.12±6.3	28.67±4.9	8.09±2.6
γδ TCR [3]	Control	39.77±5.8[a]	19.42±2.4	21.73±3.4	19.31±4.4	9.23±2.9[a]	26.18±3.8[a]	39.72±5.1[a]	14.01±5.9
	Subclinical	13.74±2.2[b]	29.29±4.9	20.19±2.7	11.75±3.5	9.11±1.8[a]	11.12±1.7[b]	22.29±4.8[b]	23.44±5.1
	Clinical	21.30±6.4[b]	22.22±8.7	23.60±10.9	22.61±8.3	24.13±5.0[b]	12.06±1.2[ab]	25.17±5.2[ab]	13.25±2.9
B-cells [4]	Control	19.43±8.1	9.32±4.3	11.96±3.9	18.47±7.7[ab]	11.04±2.3	21.66±5.4	29.41±8.0[ab]	25.26±4.5
	Subclinical	20.01±4.5	11.98±2.8	16.87±3.7	24.06±4.6[a]	24.01±2.6	24.41±2.6	24.27±4.3[a]	25.36±2.8
	Clinical	23.60±10.6	14.37±7.0	15.15±3.9	7.67±2.59[b]	14.80±5.4	9.97±2.8	18.70±4.8[b]	15.0± —

Superscripts indicate differences between infection groups within a given time point ($P < 0.05$).

[1] Infection group x day relative to calving effect: $P < 0.09$

[2] Day relative to calving effect: $P < 0.003$; Infection group x day relative to calving effect: $P < 0.02$

[3] Day relative to calving effect: $P < 0.01$; Infection group x day relative to calving effect: $P < 0.004$

[4] Day relative to calving effect: $P < 0.09$

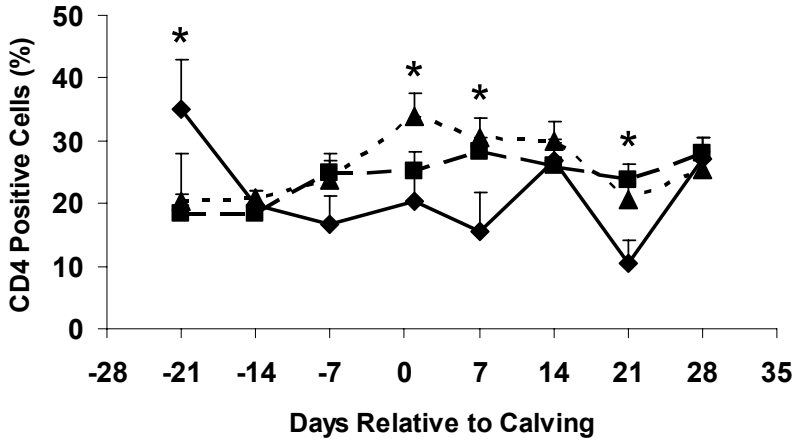
List of Figures

$CD4^+$ T-cells. Clinical cows expressed a smaller percentage compared with control cows ($P < 0.01$). **B)** $CD8^+$ T-cells. Clinical cows expressed a smaller percentage compared with control cows ($P < 0.05$). There was a tendency for a DRTC effect ($P < 0.08$). **C)** γδ T-cells. Subclinical cows expressed a lower percentage than the control cows ($P < 0.05$). **D)** B-cells. Data are least square means ± SEM. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

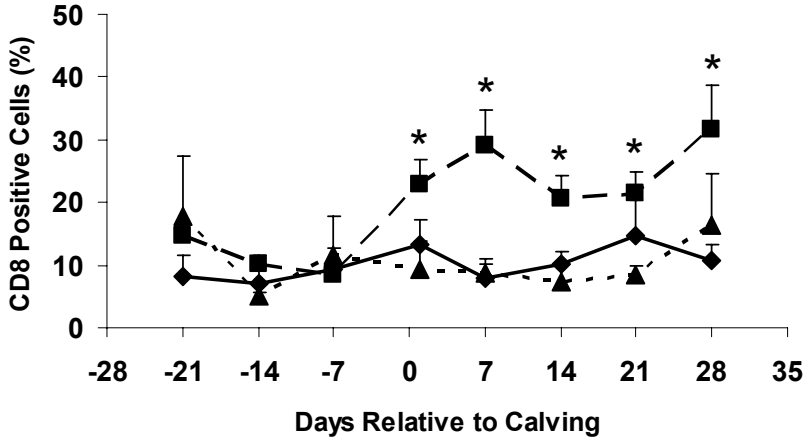
Figure 8. Percentage of $CD5^{dim}$ T-cells within lymphocyte subsets from fresh PBMCs isolated from control (♦), subclinical (■), and clinical (▲) periparturient dairy cows. **A)** $CD4^+$ T-cells. Clinical cows expressed a greater percentage compared with control cows ($P <0.05$). **B)** $CD8^+$ T-cells. Clinical cows expressed a greater percentage compared with controls ($P <0.01$) and subclinical ($P <0.06$) cows. **C)** γδ T-cells. During postpartum period, subclinical and clinical cows had greater percentage compared with controls ($P < 0.05$). **D)** B-cells. Clinical cows had greater percentage compared with controls ($P < 0.05$). Data are least square means ± SEM. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

Figure 1.

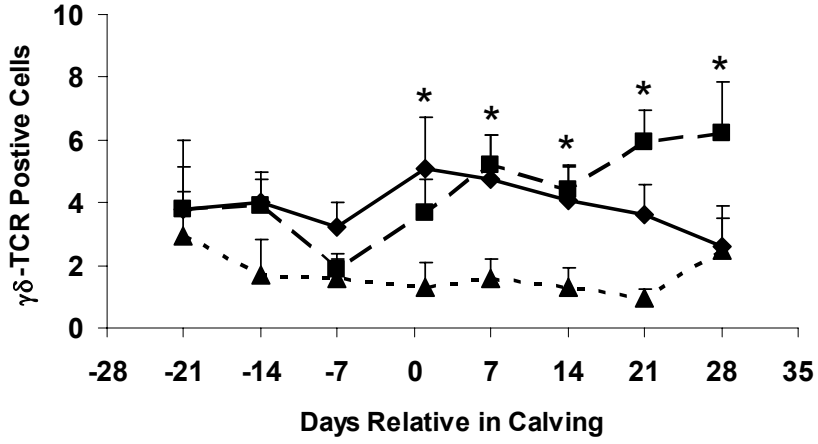
A.


CD4 Positive Cells (%)
50
40
30
20
10
0
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving

B.

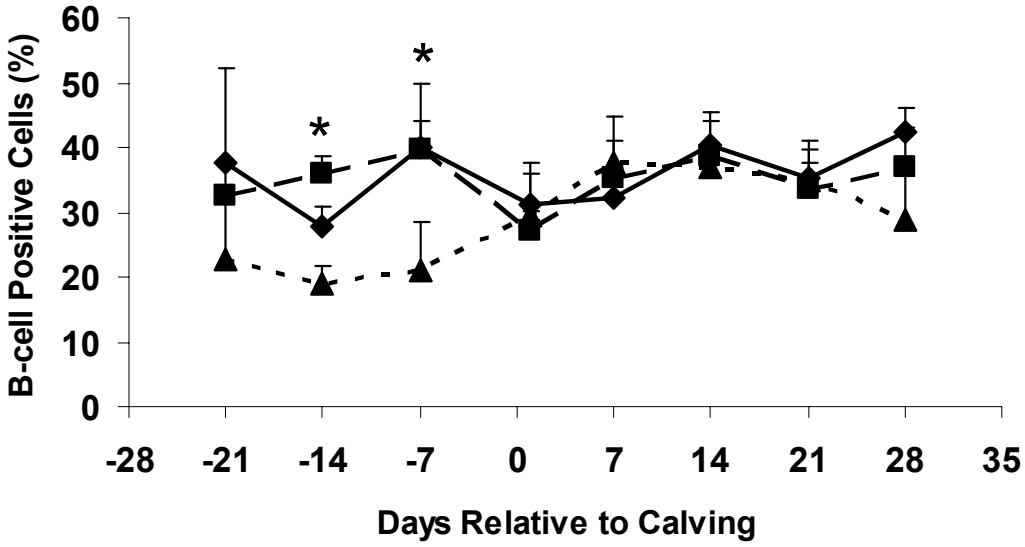

CD8 Positive Cells (%)
50
40
30
20
10
0
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving

C.


γδ-TCR Postive Cells
10
8
6
4
2
0
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative in Calving


♦ Control
■ Subclinical
▲ Clinical

Figure 2.
A.


B-cell Positive Cells (%)
60
50
40
30
20
10
0
*
*
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving

B.

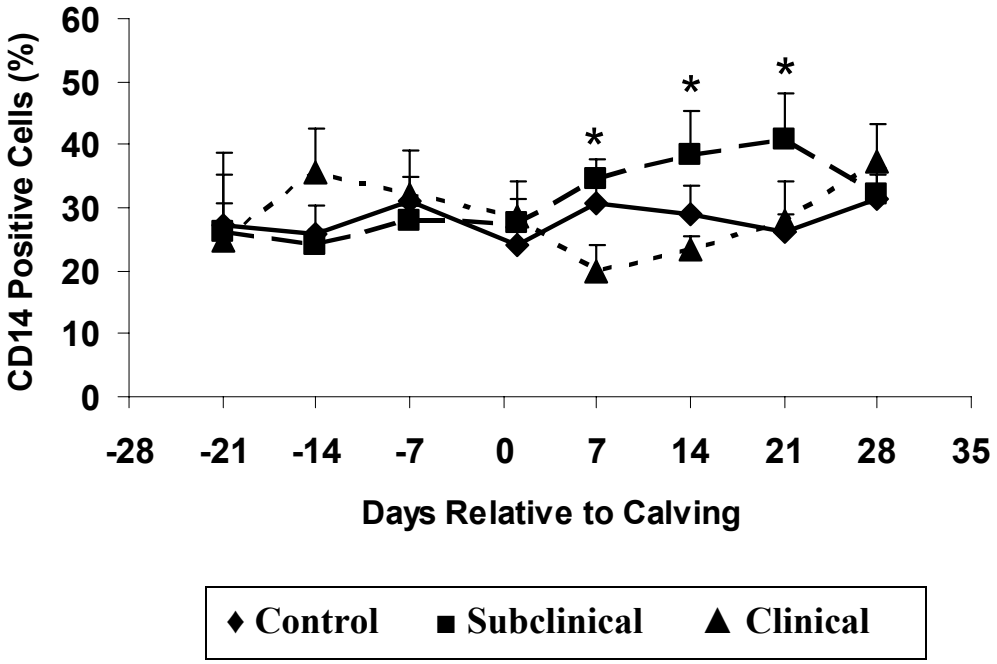

CD14 Positive Cells (%)
60
50
40
30
20
10
0
*
*
*
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving
♦ Control
■ Subclinical
▲ Clinical

Figure 3.

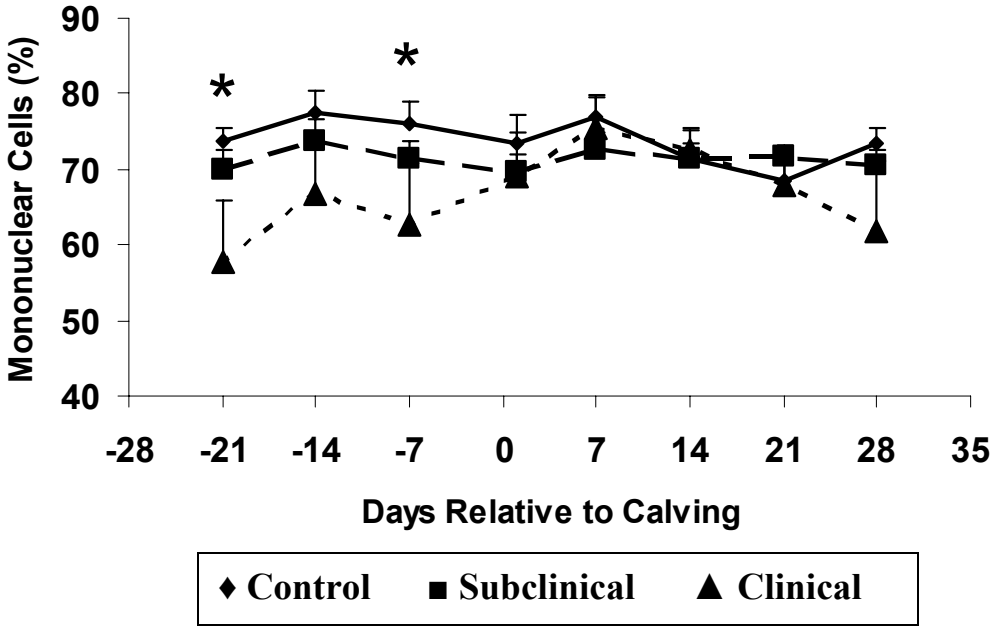
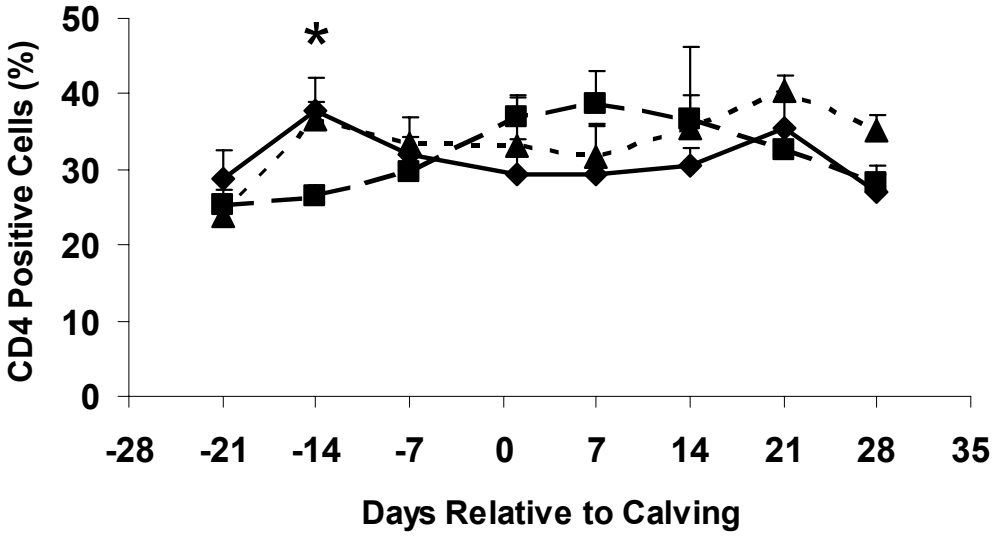

90
80
70
60
50
40
Mononuclear Cells (%)
*
*
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving
♦ Control
■ Subclinical
▲ Clinical

Figure 4.
A.


50
40
30
20
10
0
CD4 Positive Cells (%)
*
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving

B.

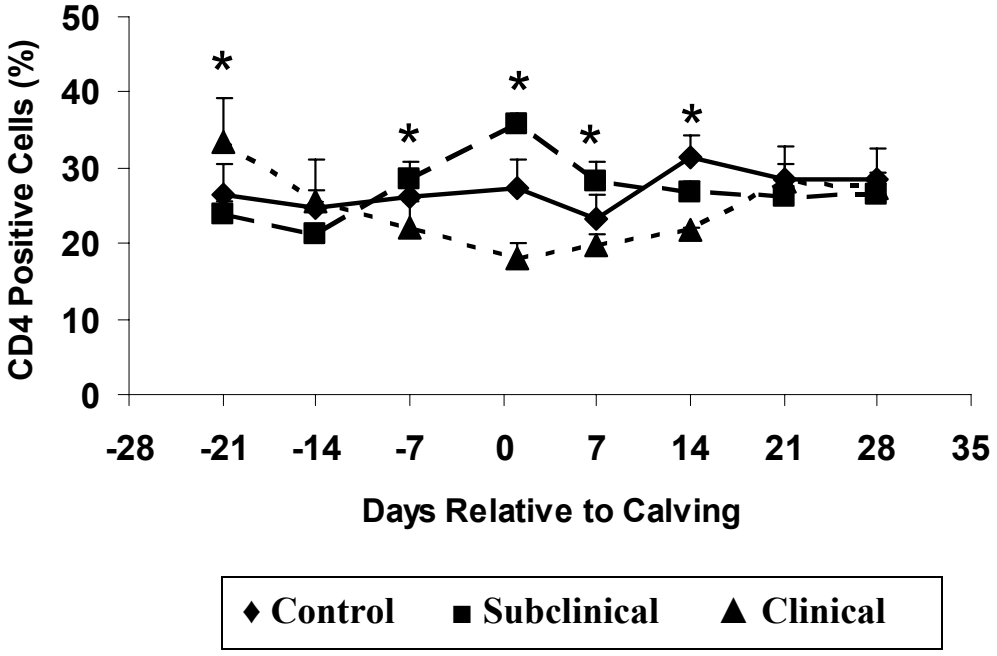

50
40
30
20
10
0
CD4 Positive Cells (%)
*
*
*
*
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving
♦ Control
■ Subclinical
▲ Clinical

Figure 5.

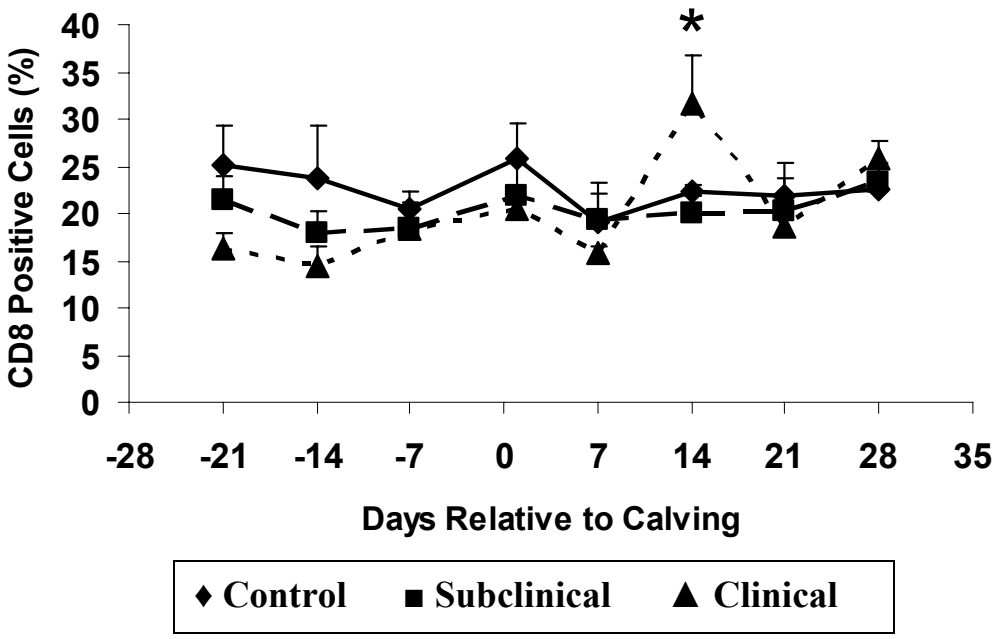

CD8 Positive Cells (%)
40
35
30
25
20
15
10
5
0
*
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving
♦ Control
■ Subclinical
▲ Clinical

Figure 6.

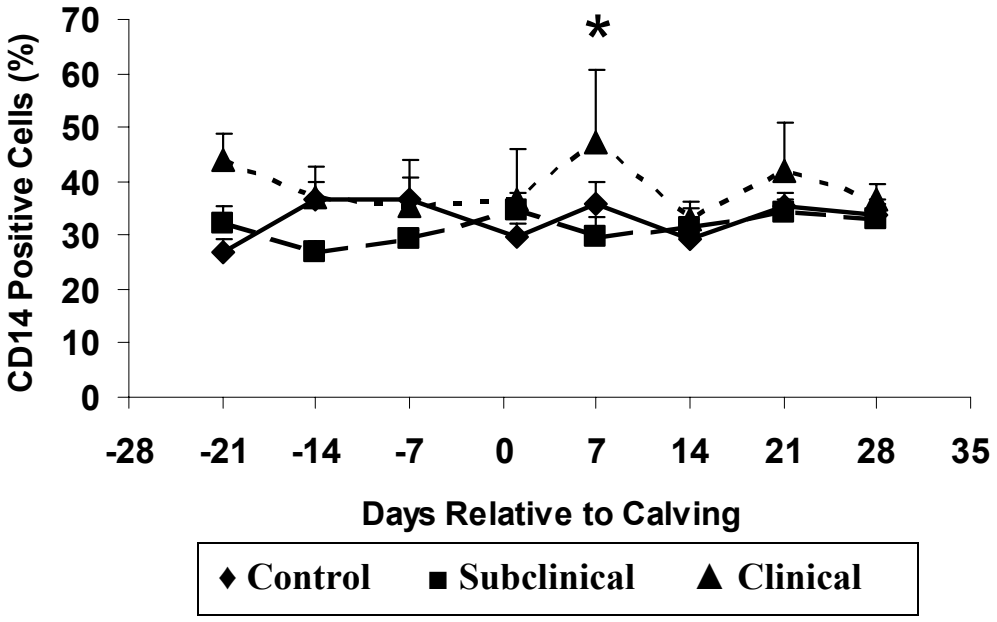

CD14 Positive Cells (%)
70
60
50
40
30
20
10
0
*
-28
-21
-14
-7
0
7
14
21
28
35
Days Relative to Calving
♦ Control
■ Subclinical
▲ Clinical

Figure 7.

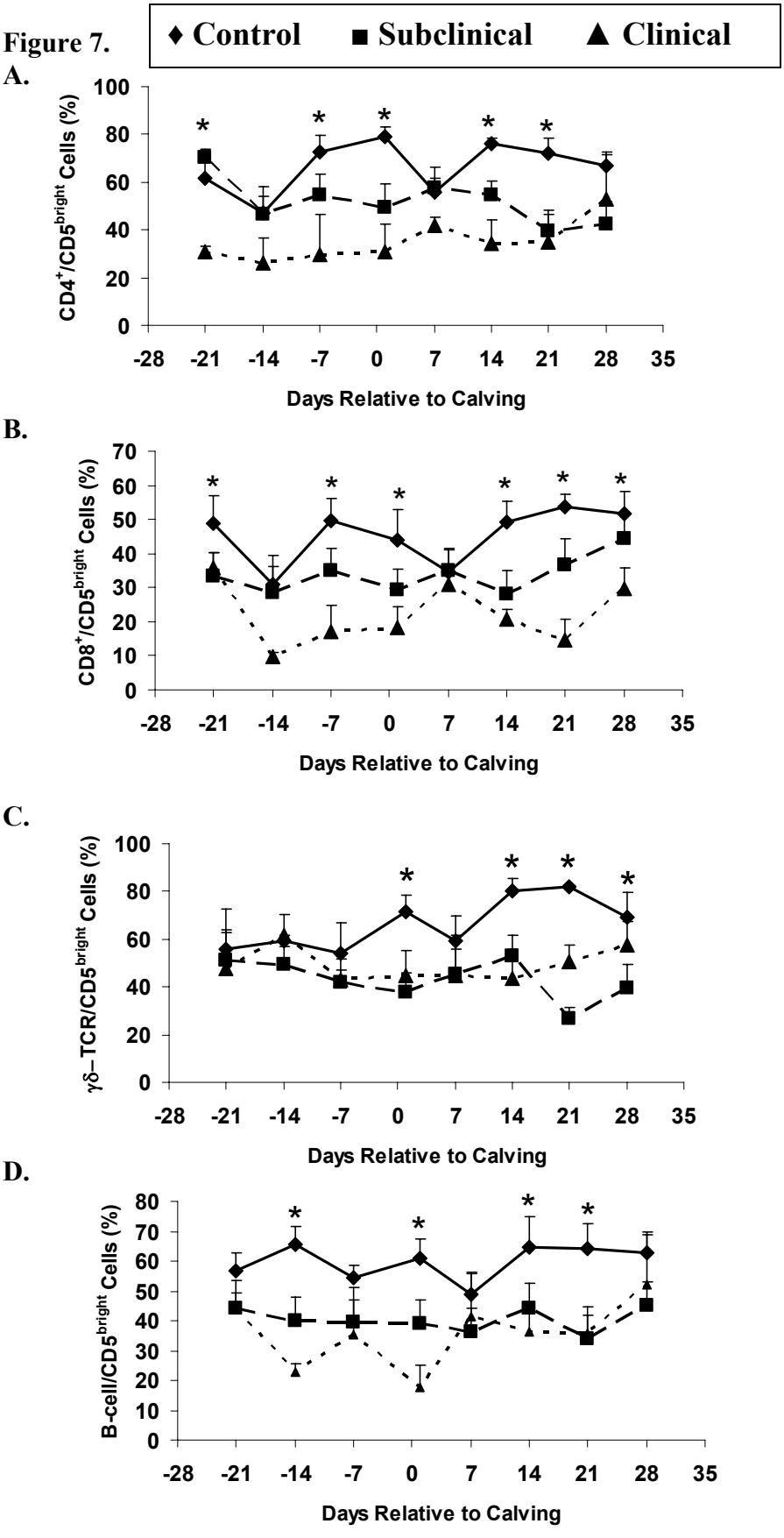
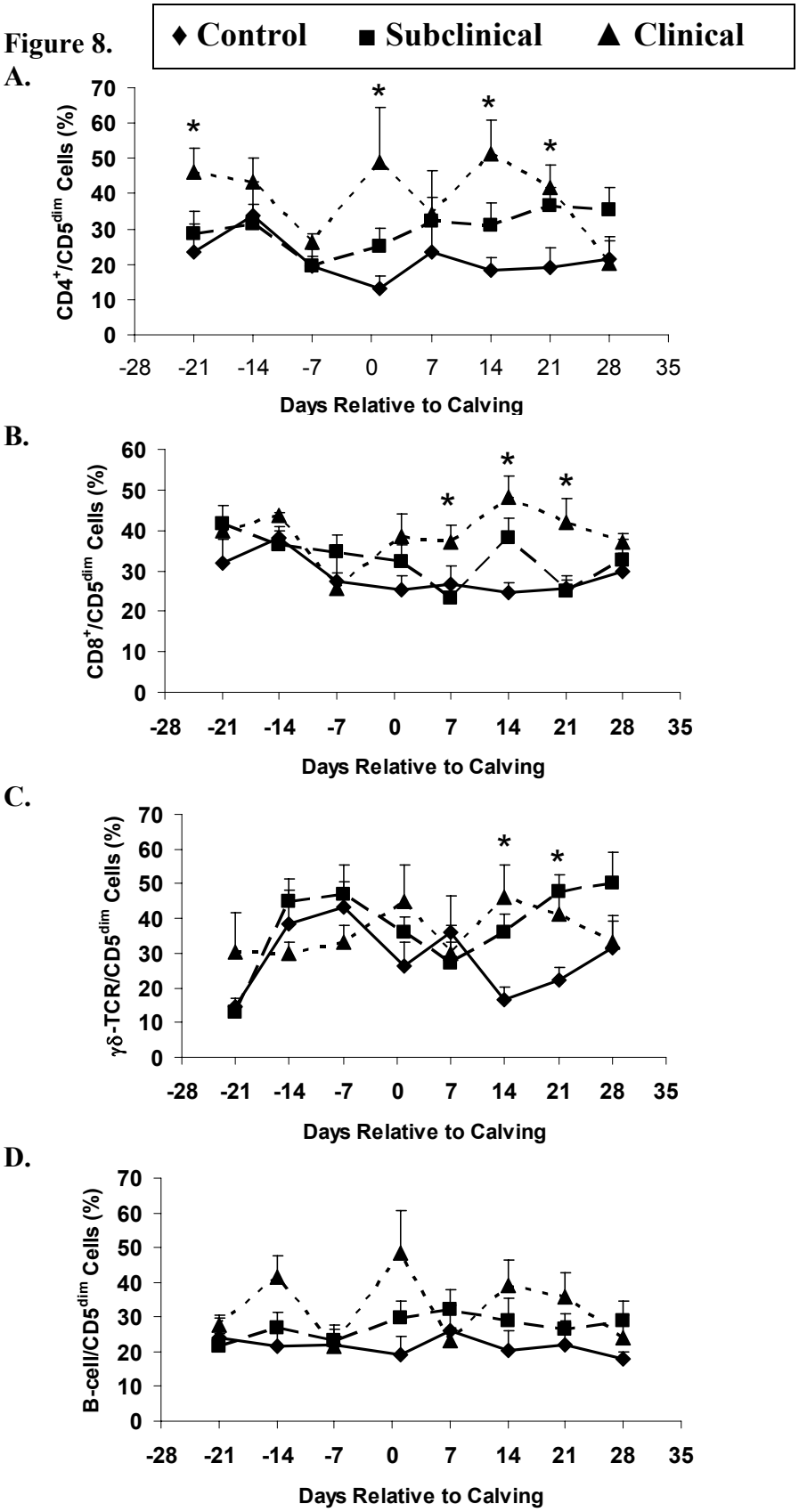

♦ Control ■ Subclinical ▲ Clinical
A.
CD4+/CD5bright Cells (%)
B.
CD8+/CD5bright Cells (%)
C.
γδ– TCR/CD5bright Cells (%)
D.
B-cell/CD5bright Cells (%)
Days Relative to Calving

Figure 8.


♦ Control
■ Subclinical
▲ Clinical
A.
CD4+/CD5dim Cells (%)
B.
CD8+/CD5dim Cells (%)
C.
γδ-TCR/CD5dim Cells (%)
D.
B-cell/CD5dim Cells (%)
Days Relative to Calving

CHAPTER FOUR

OSTEOPONTIN EXPRESSION IN PERIPARTURIENT DAIRY COWS NATURALLY INFECTED WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARAUBERCULOSIS*

A paper to be submitted to *Infection and Immunity*

E.L. Karcher[1,3], D. Bayles[2], J.P. Bannantine[2], D.C. Beitz[1], J.R. Stabel[2,4]

ABSTRACT

Osteopontin (Opn), a highly acidic glycoprotein, is an important mediator of the cell-mediated immune response and is an enhancer of the host immune response against mycobacterial infections. Infections caused by the intracellular bacteria, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), have a devastating impact on the dairy industry. We sought to characterize Opn at both the level of gene and protein expression in periparturient dairy cows naturally infected with MAP. Peripheral blood mononuclear cells (PBMCs) were isolated from control, subclinical, and clinical periparturient dairy cows naturally infected with MAP beginning 3 wks pre- to 5 wks post-calving and incubated with or without concanavalin A (ConA) or a whole-cell sonicate of MAP (MPS). Real-time PCR was performed to evaluate expression of Opn and classical Th1 and Th2 cytokines. Results demonstrated greater Opn expression in nonstimulated PBMCs isolated from subclinical cows compared with control and clinical cows. For clinical cows, there was a strong

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correlation between Opn expression and expression of the Th1 cytokines, IFN-γ and IL-1α for non stimulated PBMCs, and IFN-γ and IL-12 for PBMCs stimulated with MPS. Expression of TNF-α was greater in clinical cows than in cows of the other groups. In support of a Th1/Th2 dichotomy, NS, ConA, and MPS-stimulated PBMCs from subclinically infected cows secreted more IFN-γ and MPS-stimulated PBMCs from clinically infected cows secreted more IL-4 compared with those of the other groups. Immunoblot analysis of PBMCs detected four Opn proteins at 60, 52, 34, and 27 kDa. There was an interaction of infection group and parturition. Protein abundance increased at calving for subclinical and control cows. This is the first study to evaluate the role of Opn on the immune response of dairy cows naturally infected with MAP. Results of this study suggest Opn may be a key regulator against MAP infection.

Key Words: Osteopontin, Periparturient, *Mycobacterium avium* subsp. *paratuberculosis*

INTRODUCTION

The causative agent of Johne's disease (JD) in ruminant animals is *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Dairy cows generally are infected as neonates by the ingestion of feed or water contaminated with the organism. Once they are infected animals may remain in the subclinical or asymptomatic stage of the disease until a period of stress, such as parturition, occurs. Advancement to the clinical stage of the disease is characterized by fecal shedding of the bacteria, severe weight loss, and intermittent diarrhea. There are no known cures for JD and often the animal will succumb to the infection.

Osteopontin (Opn) is a highly acidic glycoprotein that is produced by both immune and non-immune cells, such as osteoclasts, smooth muscle cells, and epithelial cells (Denhardt and Guo, 1993). The primary immune sources of Opn are activated macrophages (Atkins et al, 1998), activated T cells (Ashkar et al., 2000), and dendritic cells (Kawamura et al. 2005). Osteopontin also is secreted from activated, but not resting, natural killer cells (Pollack et al., 1994), and it is an important mediator of the cell-mediated, or Th1, immune response. Osteopontin induces T cell chemotaxis and costimulates T cell proliferation (O'Regan et al., 2000). Stimulating murine macrophages with Opn resulted in the production of proinflammatory cytokines, IL-12 and TNF-α (Weber et al., 2002). In addition, when human gut-derived T-cells were stimulated with bovine Opn and CD3 antibody, there was a dose-dependent increase in the secretion of interferon gamma (IFN-γ) and tumor necrosis factor (TNF)-α (Agnholt et al., 2007). In addition to its effects on T-cells, Opn stimulates IgM and IgG production from B-cells (Lampe et al., 1991).

The role of Opn in mycobacterial infection is of interest because of its reported ability to upregulate and promote Th1 cytokines. When Opn knock-out mice were challenged with *M. bovis* Bacillus Calmette-Guerin, they had more severe infection, heavier bacterial loads, and greater granuloma burdens compared with the wild-type mice (Nau et al., 1999). Infecting human alveolar macrophages with *M. tuberculosis* caused an upregulation of Opn expression (Nau et al., 1997). Furthermore, a positive correlation has been noted between the amount of Opn in tissues and disease severity in patients with mycobacterial infections (Nau et al., 2000). Patients suffering from nontuberculous mycobacterial infections with high Opn protein concentrations in the lymph nodes recovered faster than did those patients with lower Opn concentrations (Nau et al., 2000).

The ability of Opn to promote a Th1 immune response and increase resistance to mycobacterial infections identified it as a cytokine of interest in the study of MAP infection in cattle. An effective Th1 response to MAP infection is critical for controlling the initial stages of the disease. Subclinical JD cows produce greater amounts of IFN-γ and TNF-α than do clinical cows (Stabel, 2000). The transition from the subclinical to clinical stage of disease coincides with a shift from Th1 to Th2-mediated host responses. Production of Th2 cytokines supports a humoral immune response by stimulating the proliferation of B lymphocytes and inhibiting Th1 cytokines. Both IL-10 and TGF-β are upregulated in clinical MAP-infected cows (Khalifeh and Stabel, 2004b).

To date, there are no reports in the literature of Opn expression in dairy cows infected with MAP. Based on this observation, and the critical role of Opn in controlling other mycobacterial infections, the objective of this study was to characterize Opn at both the level of gene and protein expression in periparturient dairy cows naturally infected with MAP. In addition, the expression and secretion of other key Th1 and Th2 cytokines was performed during this time period and correlated with Opn results.

MATERIALS AND METHODS

Animals

Twenty-five multiparous Holstein cows were grouped according to infection status. These 3 groups consisted of 8 noninfected healthy cows, 10 cows naturally infected with MAP, but asymptomatic, and 7 naturally infected cows with clinical JD. The stage of infection was determined by fecal shedding of MAP, IFN-γ secretion, and specific antibody response to MAP. Infection was monitored by bacteriologic culture for the fecal shedding of

MAP by standard methods (Stabel, 1997). By definition, clinical animals were shedding more than 100 CFU/tube (BBL™ Herrold's Egg Yolk Agar Slants with mycobactin J, amphotericin, nalidixic acid, and vancomycin; Becton, Dickinson and Co., Sparks, MD) and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 CFU/tube and were asymptomatic. The noninfected control cows were characterized by repeated negative fecal cultures performed quarterly over a 3- to 5-yr period and had been purchased from herds with no recent history of Johne's disease. In addition, these animals were negative on any serologic assays (i.e., production of antibody specific for MAP and IFN-γ) performed during that period. All procedures performed on the animals were approved by the Institutional Animal Care and Use Committee (National Animal Disease Center [NADC], Ames, IA).

Blood Collection, Culture Conditions, and Sample Collection

Blood was collected from the jugular vein in 2x acid-citrate-dextrose (ACD; 1:10). For each animal, blood was collected at -21, -14, -7, +1, +7, +14, +21, +28, and +35 days relative to calving (DRTC). Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat fractions of peripheral blood. PBMCs were resuspended in RPMI-1640 (Gibco, Grand Island, NY) with 10% fetal calf serum (Atlanta Biologics, Atlanta, GA), 100 U of penicillin G sodium per mL100 μg of streptomycin sulfate per mL, 0.25 μg of amphotericin B per mL, and 2 m*M L*-glutamine (Gibco, Grand Island, NY). Cells were cultured at 1.4 x 10^6/mL in 48-well flat-bottomed plates (Corning, Corning, NY) with either medium alone (nonstimulated**,** NS), with concanavalin A (ConA; 10 μg/mL) or with MAP whole cell sonicate (MPS;10 μg/mL) added to designated wells. Plates were incubated for 24

h at 39°C in 5% CO_2 in a humidified atmosphere. After 24 h, plates were centrifuged at 400 x g for 5 min. Supernatants were removed without disturbing the cells in culture and stored at -20°C prior to cytokine measurement.

Bacteria

M. avium subsp. *paratuberculosis* strain K-10 (NADC) was grown in Middlebrook 7H9 broth (pH 6.0) supplemented with mycobactin J (2 mg/L; Allied Monitor, Fayette, MO) and oleic acid-albumin-dextrose complex (Becton Dickinson Microbiology, San Jose, CA). The bacteria were harvested, washed 3 times with PBS (137 m*M* sodium chloride, 10 m*M* phosphate, 2.7 m*M* potassium chloride; pH 7.4), and sonicated on ice for 10 min. After incubating at RT for 10 min, the bacteria was sonicated for an additional 10 min and then centrifuged at 2500 x g for 20 min. Supernantant was removed and absorbance was read at 540 nm. Bacterial stocks (10^9/mL) were frozen in PBS at -80°C until used in the experiments.

RNA Extraction and RT-PCR

Peripheral blood mononuclear cells from each of the sampling time points were resuspended in RPMI 1640 (Gibco, Grand Island, NY) containing 10% fetal calf serum. PBMCs from each cow at each sampling time point were split into two aliquots: one aliquot that was cultured with medium alone (NS) and one that was stimulated with MPS (10 μg/mL). Cells were cultured in 75-cm^2 flasks at a concentration of 1 x 10^7 PBMCs per flask at 39°C in 5% CO_2 in a humidified atmosphere for 24 h. RNA was extracted from NS and MPS-stimulated PBMCs by using the standard protocol for Trizol Reagent (Invitrogen Life

Technologies Corp., Carlsbad, CA). All RNA samples were purified by using the RNeasy® Mini Kit Protocol for RNA Cleanup (Qiagen, Valencia, CA). Samples were treated with TURBO DNA-free (Ambion, Austin, TX). The quantity of total RNA was determined by UV spectrophotometry. RNA samples were frozen at -80°C until converted to cDNA.

Real time RT-PCR was performed by using an Applied Biosystems 7500 DNA sequence detection system (Perkin-Elmer Corp., Foster City, CA). Total RNA extracted from both NS and MPS-stimulated cells was converted to first strand cDNA. Briefly, 2 µg of total RNA was added to 12-µL reaction mixture consisting of 10 m*M* oligo(dT)$_{12-18}$ primer (Invitrogen, Carlsbad, CA) and RNase-free water. The reaction mixture was incubated at 70°C for 5 min and then chilled quickly on ice to 20°C. To the reaction mixture, 4 µL of 5x First Strand Buffer (Invitrogen, Carlsbad, CA), 2 µL of 10 m*M* dNTP Mix (Invitrogen, Carlsbad, CA), 1 µL of 0.1 M dithiothreitol (Invitrogen, Carlsbad CA), and 2 U SuperScript™ RNase H-Reverse Transcriptase (Invitrogen, Carlsbad CA) were added for a total volume of 20 µL. The reaction mixture was incubated at 42°C for 1 h, heated to 70°C for 15 min, and then cooled to 37°C. Two units of DNase-free RNase H (Invitrogen, Carlsbad, CA) were added to the mixture that then was incubated at 37°C for 20 min to remove the original RNA template. The RNase H was inactivated by heating the reaction mixture at 70°C for 10 min. All cDNA samples were stored at -80°C until RT-PCR analyses were performed.

For RT-PCR analysis, SYBR Green PCR master mixture (Perkin-Elmer Corp., Foster City, CA), template cDNA, and gene-specific primers for Opn, IFN-γ, TNF-α, IL-1α, IL-12p35, IL-4, IL-10, and β-actin were combined in a 20-µL reaction mixture. Primer sequences are listed in Table 1. All reactions were performed in triplicate. The β-actin gene

was used as the control (for calculation of dCt). RT-PCR data were analyzed by using the $2^{-(ddCt)}$ method as described previously (Livak and Schmittgen, 2001). The +1 dCt value for each cow was used as the reference expression point.

Measurement of IFN-γ, IL-10, TGF-β, and IL-4 production in cell culture supernatants by ELISA.

Bovine IFN-γ was measured by using the Bovigam test kit (CSL Veterinary Laboratories, Parkville, Victoria, Australia) as described by the manufacturer. The minimal sample concentration of IFN-γ that could be detected by using the kit was 0.39 ng/mL.

Bovine IL-10 was quantified by coating MaxiSorp microtiter plates (Nunc, Rochester, N.Y.) with mouse anti-bovine IL-10 in coating buffer (15 m*M* sodium carbonate, 34 m*M* sodium bicarbonate, pH 9.6) (100 µL per well at 2 µg/mL) (MCA2110, Serotec, Raleigh, NC) overnight at RT. Plates were washed 5 times with PBS containing 1% Tween 80 (washing buffer). The samples and serial 2-fold dilutions of bovine IL-10 standard (0.3125-20 ng/mL) (generous gift from Dr. Jayne Hope, Compton, UK) were added to duplicate wells and incubated at RT for 1 h. Plates then were washed 5 times with washing buffer before incubating with the detection antibody, mouse anti-bovine IL-10:biotin (MCA2111B, Serotec, Raleigh, NC). Plates were washed 5 times with washing buffer, 100 µL of avidin-HRP conjugate (diluted 1:800) (PharMingen, San Diego, CA) were added to each well, and the plates were incubated for 45 min at RT. After another wash cycle, plates were incubated with substrate solution (40 mM ABTS [2, 2'-azino-di-ethylbenzthiozoline-6-sulfonic acid] in citrate buffer (50 m*M*, pH 4.0) and H_2O_2 (30% solution as 1:30 dilution). Color development was quantified after 30 min by measuring absorbance at 405 nm with a Wallac Victor 1420

multilabel counter ELISA plate reader (Perkin-Elmer, Gaithersburg, MD). The minimal sample concentration of IL-10 that could be detected by using the kit was 0.3125 units.

Bovine TGF-β was quantified by using the Quantikine® Human TGF-β1 Immunoassay kit as described by the manufacturer and by using standards supplied (R&D Systems, Inc, Minneapolis, MN). Previous studies have utilized anti-bovine TGF-β1 antibodies to detect bovine TGF-β activity (Ginjala and Pakkanen, 1998; Khalifeh and Stabel, 2004a). Significant concentrations of latent TGF-β1 are found in bovine cell culture supernatants. Latent TGF-β was activated by the addition of 1 N HCl followed by 10 min of incubation. The samples were neutralized by the addition of 1.2 N NaOH/0.5 M HEPES. The concentration determined by the standard curve was multiplied by the dilution factor 1.4 to account for the activation procedure. Because of the high cost of the kit and large number of samples in this study, only NS and MPS-stimulated cell culture supernatants were evaluated at -21, -14, -7, +1, +7,+14, and +21 DRTC for this assay. The minimal sample concentration of TGF-β that could be detected by using the kit was 31.2 pg/mL. Bovine IL-4 was measured by using Bovine IL-4 Screen Set as described by the manufacturer and using standards supplied (Endogen, Rockford, IL).

Protein Extraction and Immunoblots

Peripheral blood mononuclear cells were isolated from whole blood and stored at –80°C freezer until protein extraction. At time of extraction, PBMC cell pellet was thawed on ice and 1 mL of protein extraction buffer (Tris·EDTA (pH, 8.0), 50 m*M* KCl, 1 mg of Protease Inhibitor Cocktail/mL (Sigma, St. Louis, MO)) was added. The sample then was sonicated at 25W for 30 s with a Tekmar sonic disturber (Lorton, Va.) and incubated on ice

for 10 min. Sample then was centrifuged at 2500 x g for 20 min at 4°C and supernatant transferred to 1.5 mL microcentrifuge tube. Protein was quantified by using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

Protein samples were incubated at 95°C for 5 min and separated (16 µg of protein per lane) by 4-20% SDS polyacrylamide gel electrophoresis (Laemmli, 1970). The separated proteins were electrotransferred to 0.45-µm pure nitrocellulose membrane. For analysis of Opn and β-actin proteins, the membrane was blocked by overnight incubation in PBS plus 2% bovine serum albumin (BSA) and 0.1% Tween 20 (PBS-BSA). The membrane then was incubated at RT for 2 h in PBS-BSA containing a polyclonal anti-mouse Opn (1:2500 dilution; Cosmo Bio Co., LTD, Tokyo) antibody. After three washes in PBS plus 0.1% Tween 20, blots were incubated for 1.5 h in protein A-peroxidase (1:20,000 dilution; Pierce Biotechnology Inc., Rockford, IL). The blots again were washed 3x as described above and were developed for chemiluminescence by using Supersignal detection reagents (Pierce Biotechnology Inc., Rockford, IL). The membranes were blotted gently, wrapped in plastic wrap, and exposed to x-ray film (Kodak X-Omat AR, Rochester, NY). Membranes then were stripped by incubating in Restore Western Blot Blocking Stripping Buffer (Pierce Biotechnology, Rockford, IL) for 15 min at RT and then washing 3 x for 5 min each in PBST. To insure the membrane had been stripped completely, the membrane was exposed to x-ray film (Kodak X-Omat AR, Rochester, NY). Membranes then were blocked by overnight incubation in PBS plus 2% PBS-BSA and the following day re-probed for β-actin (1:2500 dilution; Imgenex, San Diego, CA).

Determination of Molecular Size and Relative Protein Abundance

Digital images of the autoradiographic film from the immunoblots were scanned, and scanning densitometry was performed with the Kodak Digital Science 1-D Image Analysis Software (Eastman Kodak Co., Rochester, NY). Relative molecular weights of Opn and β-actin protein were calculated by regressing the distance of migration of the protein through the gel, against the relative molecular weights of known markers ranging from 20.9 to 101 kDa (Prestained SDS-PAGE Standards, low range; BioRad, San Diego, CA).

Statistical Analysis

RT-PCR data were analyzed by using the $2^{-(ddCt)}$ method as previously described (Livak and Schmittgen, 2001). β-actin was used as the reference gene, and the dCt value at +1 d for each animal was used as the reference expression point. Outliers were determined by the SAS/STAT PROC RobustReg software. To evaluate the appropriateness of β-actin as a reference gene, the $2^{-(dCt)}$ method was used as previously described (Schmittgen and Zakrajsek, 2000). This test indicated that β-actin would serve as a suitable reference for both healthy and MAP-infected animals.

Several 2-factor repeated measures analyses of variance were performed (one for each stimulation level-gene combination) comparing fold expression of the treatments through time. The model accounted for infection group, parturition, and the interaction of infection group and parturition. Levene's homogeneity of variance test was performed on data to determine transformation necessity. All analyses were performed on transformed data where necessary, but raw data means are presented for ease of interpretation. If a significant F-test value from the ANOVA was obtained at $P \leq 0.05$, differences of least

squares means were used as the pairwise multiple comparison test for determining day or infection group x day differences. Means differed if $P < 0.05$ and tended to differ if $0.05 \leq P \leq 0.15$. Analyses were performed by using PROC MIXED in SAS® PC Windows Version 9.1.3 software. Pearson correlation coefficients were obtained by using the PROC CORR procedure of SAS.

RESULTS

Osteopontin Gene Expression

Overall, expression of Opn from NS PBMCs was greater ($P < 0.05$) in subclinically infected animals compared with control and clinical cows (Fig. 1A). There was also a significant interaction of infection group and parturition ($P < 0.01$). For the subclinical cows, Opn expression increased during the postpartum period and was significantly greater ($P < 0.05$) at +14, +28, and +35 d compared with +1 d. Osteopontin expression in NS PBMCs was reduced 7.7-fold between -21 and +1 d ($P <0.001$) in control cows and 6.9-fold between -7 d and +1 d ($P <0.01$) in clinical cows.

When PBMCs were stimulated with MPS, there was an overall effect of infection group, with control cows expressing more Opn than subclinical ($P < 0.001$) and clinical ($P < 0.05$) cows (Fig. 1B). For subclinical cows, expression increased ($P < 0.001$) at calving and then declined during the postpartum period. In contrast, a decline ($P < 0.03$) in Opn expression by MPS-stimulated PBMCs was observed for clinically infected cows as calving approached followed by an increase during the postpartum period.

Effect of Infection Status on Gene Expression

Overall, expression of the Th1 cytokines, IFN-γ, IL-12, IL-1α, for both NS and MPS-stimulated PBMCs, was not affected by infection status of the cows. The expression of TNF-α by NS PBMCs was increased significanly in clinical cows (6.39 ± 1.2) compared with control (2.19 ± 1.0; $P < 0.02$) and subclinical cows (1.86 ± .9; $P < 0.001$) (Fig. 2A). There was no effect of infection on TNF-α from MPS-stimulated PBMCs (Fig. 2B). NS PBMCs from control cows tended ($P < 0.10$) to express more IL-1α compared with those from infected cows (Fig. 2C). In contrast, after stimulation with MPS there was a tendency ($P < 0.13$) for PBMCs isolated from subclinical cows to express more IL-1α compared with those from control cows (Fig. 2D).

There was a lack of an overall effect of infection on the expression of Th2 cytokines, IL-4 and IL-10. However, MPS-stimulated PBMCs from control cows expressed more IL-4 than those from subclinical cows ($P < 0.05$) and tended to express more than those from clinical cows ($P < 0.09$) (Fig. 3A). The expression of IL-10 from NS PBMCs tended to be greater ($P < 0.10$) for control cows compared with that of clinical cows (Fig 3B) whereas, MPS-stimulated PBMCs from subclinical cows expressed more ($P < 0.05$) IL-10 compared with those from clinical cows (Fig. 3C).

Effect of Parturition on Gene Expression

There was no effect of parturition on expression of IL-12 or IL-1α for NS and MPS PBMCs. There was an overall effect of parturition ($P < 0.01$) on the expression of IFN-γ from NS PBMCs (Fig. 4). Expression by clinical cows declined ($P < 0.01$) from -7 d to +21

d with a brief rebound by +28 d (P <0.01). Interferon expression by subclinical cows decreased (P < 0.06) from -7 d to +1 d. There was also a significant effect of parturition (P < 0.01) on expression of TNF-α by NS PBMCs (Fig. 2A). Control cows experienced a decline (P < 0.05) in TNF-α expression during the last week of the prepartum period. Expression of TNF-α for NS PBMCs from clinical cows declined (P < 0.001) from -21 d to +21 d, with a rapid increase (P < 0.001) at +28 d. Similarly, expression of TNF-α by MPS-stimulated PBMCs declined (P < 0.04) in clinical cows from -14 d to +1 d (Fig. 2B).

There was no overall effect of parturition on the expression of IL-4 or IL-10 by NS or MPS-stimulated PBMCs. However, expression of IL-10 by NS PBMCs isolated from control cows declined (P < 0.02) from -21 d to +1 d and then increased (P < 0.02) to +35 d (Fig. 3B).

Measurement of IFN-γ, IL-10, IL-4, and TGF-β production in cell culture supernatants by ELISA

Production of IFN-γ by NS (Fig. 5A) and ConA-stimulated (Fig. 5B) PBMCs tended to be greater (P < 0.08) in subclinical cows compared with the control cows. Secretion by subclinical cows increased (P < 0.05) from -21 d to +7 d. Production of IFN-γ by MPS-stimulated PBMCs, was greater (P < 0.01) in subclinical cows compared with control cows and tended to be greater (P < 0.11) than clinical cows (Fig. 5C). In subclinical cows, secretion increased (P < 0.002) from -7 d (1.24 ng/mL ±0.4) to +7 d postpartum (4.2 ng/mL ± 2.2).

Interleukin-10 secretion by NS PBMCs was not affected by infection group or parturition (Fig. 6A). However, subclinical cow secretion of IL-10 increased (P < 0.01) from

-21 d to +1 d and then declined ($P < 0.02$) to +7 d. Secretion also increased ($P < 0.04$) for control cows from -14 d to +7 d returning to precalving levels in the postpartum period. When stimulated with ConA, secretion was greater ($P < 0.003$) in subclinical cows compared with controls and tended to be greater ($P < 0.09$) than that of clinical cows (Fig. 6B). A significant effect of parturition ($P < 0.03$) also was observed. Secretion of IL-10 in subclinical cows steadily increased ($P < 0.0001$) from -21 d (0.72 U± 0.2) to +35 d (3.87 U± 0.9). Production of IL-10 by MPS-stimulated PBMCs was greater ($P < 0.04$) in subclinical cows compared with controls (Fig. 6C). There was also an effect of parturition with IL-10 secretion from control ($P < 0.04$), subclinical ($P < 0.001$), and clinical ($P < 0.05$) cows increasing from -21 d to +14 d.

Interleukin-4 secretion by NS PBMCs tended to be greater ($P < 0.11$) in clinical cows compared with controls (Fig. 7A). There was an infection by parturition interaction on IL-4 secretion by PBMCs ($P < 0.01$). Secretion by clinical cows increased ($P < 0.03$) from -21 d to +1 d. When cells were stimulated with ConA, an interaction of infection group and parturition was observed ($P < 0.03$) (Fig. 7B). Interleukin-4 secretion by MPS-stimulated PBMCs was greater in clinical cows compared with control ($P < 0.004$) and subclinical cows ($P < 0.04$) (Fig. 7C). Secretion of IL-4 by clinical cows increased ($P < 0.02$) from -21 d to +7 d and control cow secretion increased ($P < 0.04$) from -14 d to +7 d.

Secretion of TGF-β by NS PBMCs was not affected by infection group (Fig. 8A), however, there was an affect of parturition ($P < 0.01$) and an overall infection group x parturition interaction ($P < 0.03$). There was a tendency ($P < 0.15$) for subclinical (1793.2 ± 35.3) and clinical (1795.7 ± 42.9) cows to have increased secretion compared with controls (1708.8 ± 38.8). Secretion of TGF-β by PBMCs from subclinical cows increased ($P <$

0.0001) from +1 d to +21 d (1639.2 ± 73.8 vs. 1921.9 ± 75.2). Secretion by PBMCS from clinical cows rapidly declined ($P < 0.01$) from -21 d to +1 d and then increased ($P < 0.04$) from +1 d to +21 d ($P < 0.04$). Stimulation of PBMCs with ConA did not result in an overall effect of infection group or parturition on TGF-β secretion (Fig. 8B). However, secretion from subclinical cows increased ($P < 0.05$) from -7 d to +1 d and then declined until +7 d. Secretion from clinical cows declined ($P < 0.05$) from -14 d to +14 d (1795.9 ± 99.3 vs. 1590.4 ± 51.6). Secretion of TGF-β by MPS-stimulated PBMCs was not affected by infection group or parturition (Fig. 8C).

Osteopontin Protein Analysis

The immunoblotting procedure detected Opn protein bands at 24, 37, 50, and 62-kDa in the PBMC lysates of all animals (Fig. 9A). Purified bovine milk Opn was used as a standard and is identified as a single band at 60 kDa. The densities of the 24, 37, and 62-kDa proteins varied extensively between cows over the periparturient period and the variation did not seem to be related to infection group. Densitometry software was unable to consistently detect these three proteins. The 50-kDa protein was consistently the most intense of the four detected isomers. Therefore, to determine the relative abundance of Opn total protein, the 50-kDa band was analyzed. Sample dates were grouped into precalving (-21 d, -14 d, and -7 d), calving, and postcalving (+7 d, +14 d, +21 d, and +28 d). Analysis of total Opn protein abundance by immunoblotting revealed an interaction of infection group and parturition ($P < 0.01$) (Fig. 9B). The protein abundance in PBMCs from control cows increased throughout the sampling period ($P < 0.02$). PBMCs isolated from subclinical cows had an increase in protein abundance at calving and then declined during the postpartum period ($P < 0.06$).

DISCUSSION

Osteopontin, also identified as early T cell activator-1, is a highly acidic glycoprotein that is produced by a number of cells in the immune system. Within the immune system, Opn is produced by activated macrophages (Atkins et al., 1998), activated $CD4^+$ T-cells (Ashkar et al., 2000), and dendritic cells (Kawamura et al., 2005). The ability of Opn to upregulate and promote pro-inflammatory cytokines in response to intracellular bacterial infections made it an interesting candidate to evaluate in MAP-infected dairy cows.

Many calves will become infected with MAP during the first six months of life (Sweeney, 1996) through the fecal-oral route. Once infected, the cow often remains in the subclinical, or asymptomatic, stage of the disease until a period of heightened stress occurs. The subclinical stage of paratuberculosis is characterized by a Th1- or cell-mediated immune response. In the current study, there was an increase in Opn expression in subclinical cows compared with expression in control and clinical cows. This result is important because an increase in Opn expression supports the Th1 host response typically observed in subclinically infected dairy cows. Osteopontin enhances the production of other Th1 cytokines, including IL-12 and TNF-α (Ashkar et al., 2000; Weber et al., 2002). IL-12 regulates the balance of Th1 and Th2 cells by promoting the differentiation of naïve T cells to Th1 cells. Surprisingly, IL-12 expression was not affected by either MAP infection or parturition in this study. Previous reports have demonstrated that subclinical JD cows have greater expression and secretion of IFN-γ, another Th1 cytokine, compared with that of clinical cows (Stabel, 2000; Khalifeh and Stabel, 2004). Although IFN-γ expression was not affected by infection status in the present study, consistent with the literature subclinical cows secreted greater

quantities of IFN-γ compared with control and clinical cows. Along with IFN-γ, TNF-α is involved in the early stages of mycobacterial infections by controlling bacterial proliferation (Appelberg, 1994). Interestingly, in this study NS PBMCs from clinical cows expressed more TNF-α then the controls or subclinical animals.

The importance of the relationship between Opn and the Th1 cytokines was further emphasized when assessing the Pearson correlation data. Interesringly, significant correlations were observed only for the clinically infected cows. In MPS-stimulated PBMCs isolated from clinical cows, the Pearson correlation coefficient for Opn and IFN-γ expression was 0.76 ($P < 0.0001$) and 0.50 for Opn and IL-12 expression ($P < 0.004$), whereas, in the NS PBMCs from clinical cows, the coefficient for Opn and IFN-γ expression was 0.53 ($P < 0.0003$) and for Opn and IL-1α expression was 0.52 ($P < 0.001$). The correlations presented here are important because they support a positive relationship between Opn and key Th1 cytokines in MAP-infected cattle.

Typically in MAP infection, as the disease progresses from a subclinical to a clinical stage there is a transition from the Th1 to Th2 immune response. This transition has been documented by the upregulation of IL-10 and TGF-β in ileal tissues from clinical JD cows (Khalifeh and Stabel, 2004). In addition, Coussens et al. (2004) reported decreased expression of IL-4 from PBMCs isolated from subclinical JD cows compared with clinical and control cows. In the present study, the secretion of IL-4 by NS and MPS-stimulated PBMCs was greater in clinical cows compared with the control and subclinical cows. This finding is consistent with the transition from a Th1 to Th2 response occurring in clinical JD cows. TGF-β and Th2 cytokines work synergistically to inhibit the secretion of IFN-γ and the activation of macrophages. However, Opn is capable of inhibiting the expression of Th2

cytokines. Mice deficient in Opn expression had increased IL-10 production and decreased macrophage function (Ashkar et al., 2000). In the current study, TGF-β secretion for both subclinical and clinical dairy cows rapidly increased at calving and continued to increase until +21 d. This increase at calving in subclinical cows mirrored a decline in Opn expression. This is the first study to report an interaction of the Th3 cytokine, TGF-β and Opn in dairy cattle.

Published reports indicate that the expression and secretion of IFN-γ begins to decline as parturition approaches (Ishikawa et al., 1994; Shafer-Weaver et al., 1999). The decline in Opn as parturition approached further supports the classification of Opn as a Th1 cytokine. In late gestation, there is a shift in the $CD4^+$ T-cell population supporting a Th2 response in healthy dairy cows (Shafer-Weaver et al., 1999). In the present study, IL-10 secretion from all groups, regardless of infection status, increased during the last week of gestation. Furthermore, clinical cows showed increased expression of IL-4 from -21 d to +7 d post-calving. Together, these results support a Th2 response during the immediate prepartum and postpartum period.

Despite a lack of studies evaluating the role of Opn in MAP infection, there are an extensive number of human and murine studies documenting interactions of other mycobacterial infections and Opn. When Opn knock-out mice were challenged with 10^7 CFU of *M. bovis* Bacillus Calmette-Guerin, the mutant mice had more severe infection, heavier bacterial loads, and greater granuloma burdens compared with the controls (Nau et al., 1999). Another study utilizing Opn knock-out mice demonstrated a 95% and 90% decrease in IL-12 and IFN-γ secretion, respectively, compared with the wild-type controls (Ashkar et al., 2000). These knock-out studies present convincing data that establish the

importance of Opn involvement in the immediate host immune response to mycobacterial challenges.

This is the first study to report protein expression of Opn in PBMCs isolated from Holstein dairy cows. We identified four isomers of the Opn protein at 24, 37, 50, and 62 kDa. The 50 kDa protein was dominant for all animals, but there was extensive cow-to-cow variation in the 24, 37, and 62 kDa isomers. Multiple isomers of the Opn protein are not uncommon in tissues. Three Opn isomers at 60, 40, and 22 kDa were identified in the testicular parenchyma of the bull reproductive tract (Erikson et al., 2007) and using an antibody to bovine milk Opn, Kimmins et al. (2004) identified Opn proteins at 70, 45, 34, and 24 kDa in cyclic bovine endometrium. It has been suggested that the lower molecular weight isomers (20-25 kDa) are part of the COOH-terminal portion of Opn (Zhang et al., 1990). The active form of Opn in bovine PBMCs is unknown, however, a 45 kDa Opn protein was detected in human monocyte/macrophages after treatment of cells with IFN-γ and LPS (Li et al., 2003). There is a subset of T-cells that secrete a 40 kDa Opn protein that is capable of activating B cells to produce immunoglobulins (Leibson et al., 1981). Osteopontin protein abundance by PBMCs from control cows increased throughout the sampling period. Subclinical cows had an increase in Opn abundance at calving and then a decline in the postpartum period. An increase in Opn at calving was not consistent with a decline in Th1 cytokines as parturition approached.

CONCLUSIONS

This study presents the first known data examining Opn gene and protein expression in periparturient dairy cows naturally infected with MAP. Results of this study indicate that

in dairy cows expression of Opn is modulated by natural infection with MAP and by the periparturient period. Furthermore, the present study demonstrates for the first time the presence of Opn protein expression in PBMCs of healthy and MAP-infected dairy cows. Our data suggest that Opn may be a key regulator in MAP infection.

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Table 1. Primers used for RT-PCR of bovine cytokines

Gene	Primer[1]	Sequence
β-actin	F	CGCCATGGATGATGATATTGC
β-actin	R	AAGCCGGCCTTGCACAT
IFN-γ	F	TGGAGGACTTCAAAAAGCTGATT
IFN-γ	R	TTTATGGCTTTGCGCTGGAT
IL-1α	F	TTGGTGCACATGGCAAGTG
IL-1α	R	GCACAGTCAAGGCTATTTTTCCA
IL-4	F	GCCACACGTGCTTGAACAAA
IL-4	R	TGCTTGCCAAGCTGTTGAGA
IL-10	F	GCCTTGTCGGAAATGATCCA
IL-10	R	TCAGGCCCGTGGTTCTCA
IL-12p35	F	CTTTCTTCAAATGCAGCATTGG
IL-12p35	R	GGGTCTGGGTGATACAACGAA
Opn	F	ACAGCCAGGACGTCAACTCT
Opn	R	GTGAGACTCGTCGGAATGGT
TNF-α	F	TCTACCAGGGAGGAGTCTTCCA
TNF-α	R	GTCCGGCAGGTTGATCTCA

[1]F, forward; R, reverse.

List of Figures

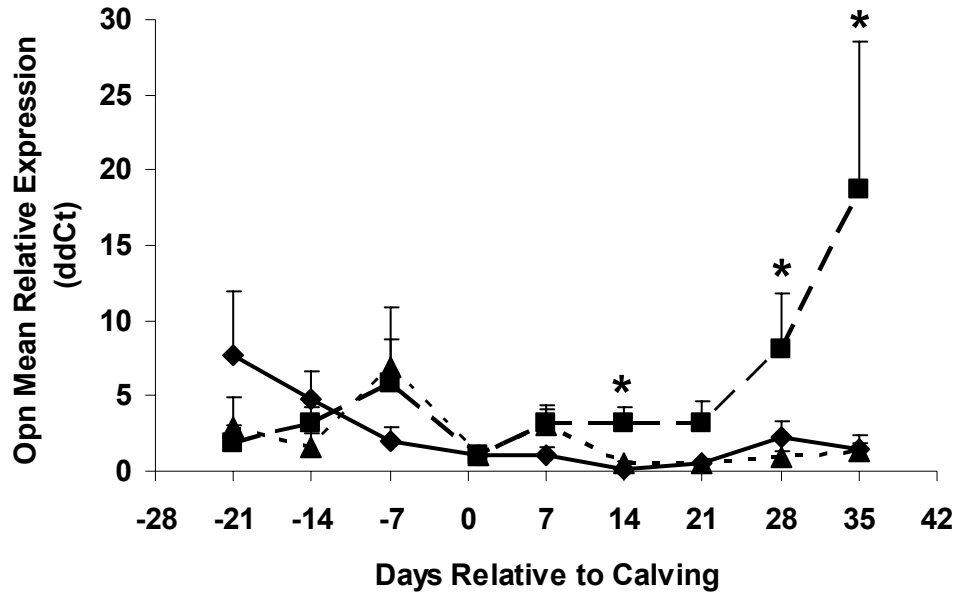
A) NS PBMCs. (Infection group and Parturition interaction, $P < 0.01$) B) ConA PBMCs. (Infection group and Parturition interaction, $P < 0.03$) C) MPS PBMCs. (Infection group, $P < 0.05$; Parturition effect, $P < 0.0002$). Significant differences between infection groups on a given day represented by asterisks ($P < 0.05$).

Figure 8. TGF-β secretion from peripheral blood mononuclear cells isolated from control (♦), subclinical (■), and clinical (▲) periparturient dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. Cells were incubated in RPMI for 24 hours. A) NS PBMCs. (Parturition, $P < 0.01$; Infection group and Parturition interaction, $P < 0.03$) B) ConA PBMCs. C) MPS PBMCs. Significant differences between infection groups on a given day represented by asterisks ($P < 0.05$).

Figure 9. Osteopontin protein expression in PBMCs from peripheral blood mononuclear cells isolated from whole blood of control (♦), subclinical (■), and clinical (▲) periparturient dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. **A)** Detection of Osteopontin protein using Western Blot Analysis. Picture is of a representative blot from one subclinically MAP-infected periparturient dairy cow across the sampling time period. **B)** Densitometric analysis of protein relative abundance compared with calving. (Infection group and Parturition interaction, $P < 0.01$).

Figure 1

A.


Opn Mean Relative Expression (ddCt)
30
25
20
15
10
5
0
-28
-21
-14
-7
0
7
14
21
28
35
42
Days Relative to Calving
*
*
*

B.

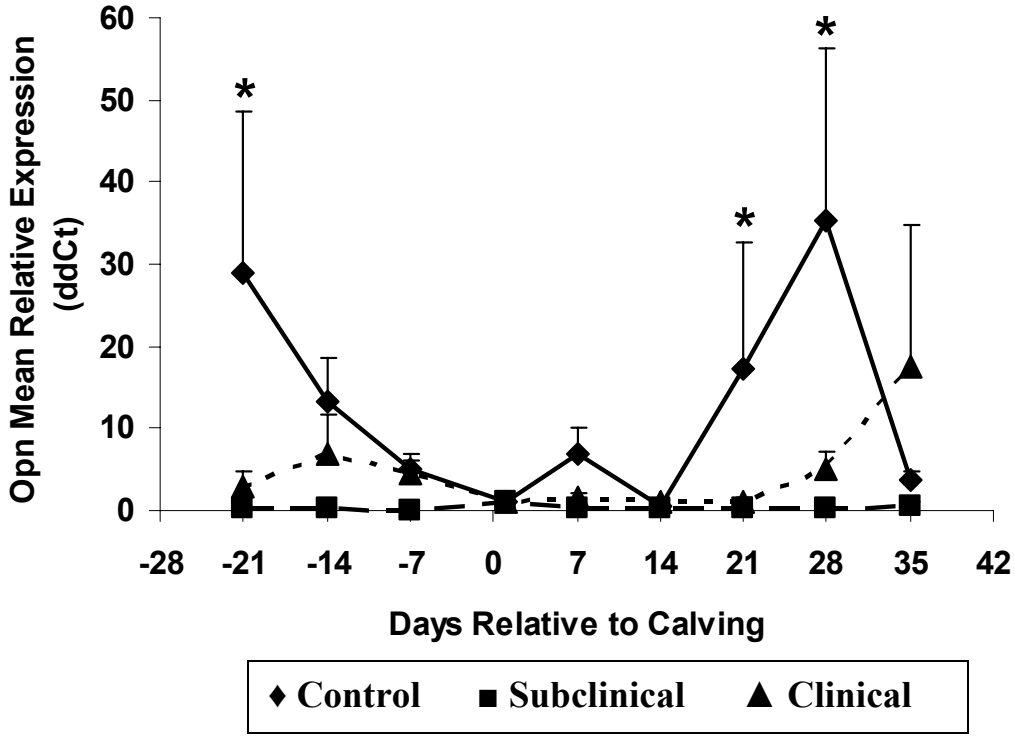

Opn Mean Relative Expression (ddCt)
60
50
40
30
20
10
0
-28
-21
-14
-7
0
7
14
21
28
35
42
Days Relative to Calving
*
*
*
♦ Control
■ Subclinical
▲ Clinical

Figure 2

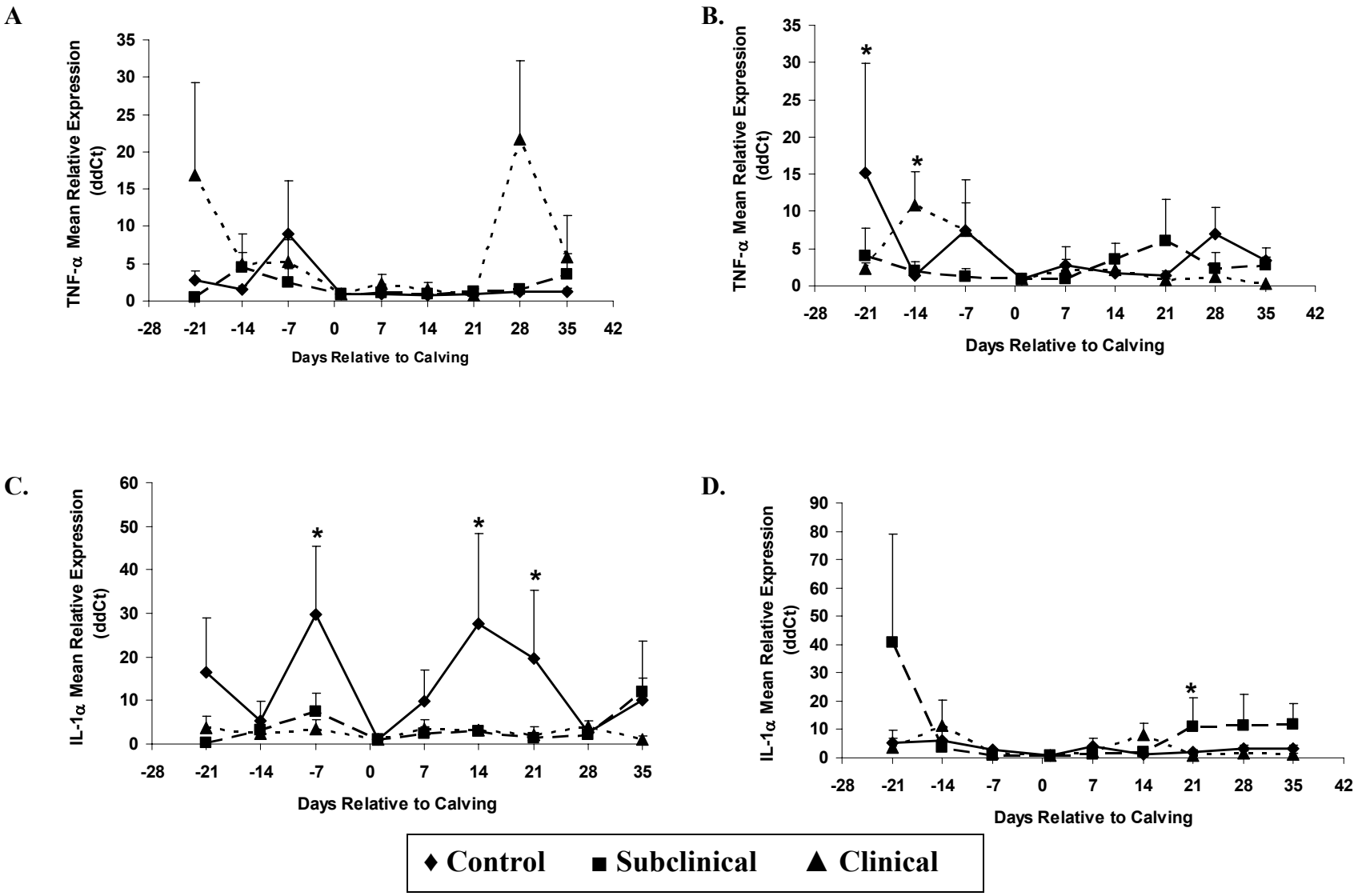
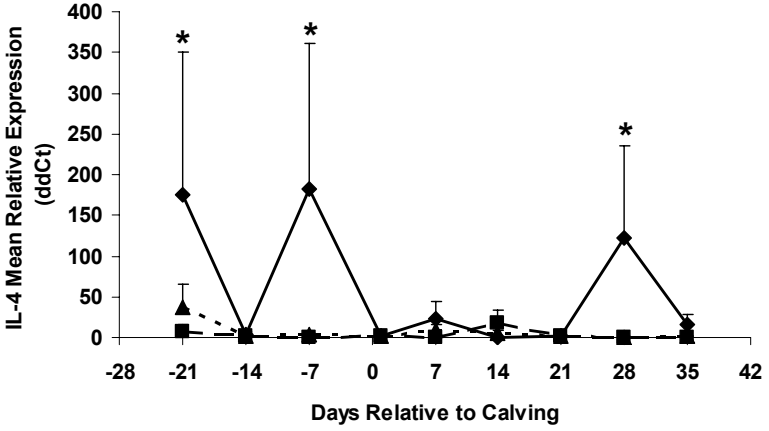
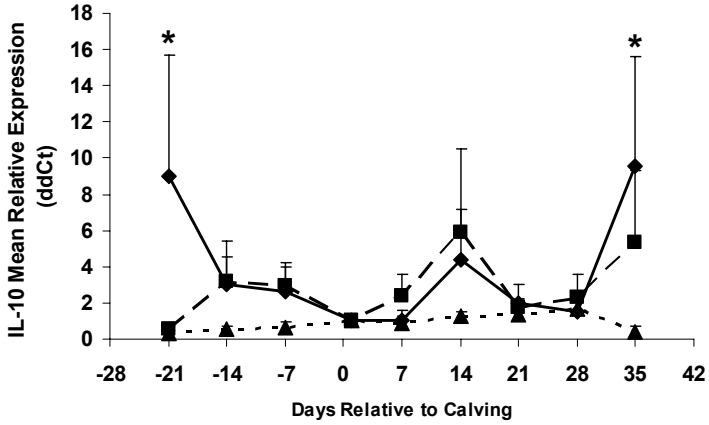

A
TNF-α Mean Relative Expression (ddCt)
Days Relative to Calving
B.
TNF-α Mean Relative Expression (ddCt)
Days Relative to Calving
C.
IL-1α Mean Relative Expression (ddCt)
Days Relative to Calving
D.
IL-1α Mean Relative Expression (ddCt)
Days Relative to Calving
♦ Control ■ Subclinical ▲ Clinical

Figure 3

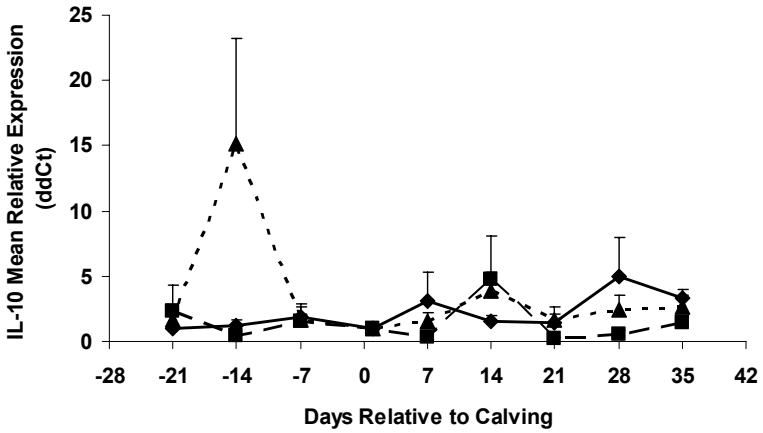
A.


IL-4 Mean Relative Expression (ddCt)
400
350
300
250
200
150
100
50
0
*
*
*
-28
-21
-14
-7
0
7
14
21
28
35
42
Days Relative to Calving

B.


IL-10 Mean Relative Expression (ddCt)
18
16
14
12
10
8
6
4
2
0
*
*
-28
-21
-14
-7
0
7
14
21
28
35
42
Days Relative to Calving

C.


IL-10 Mean Relative Expression (ddCt)
25
20
15
10
5
0
-28
-21
-14
-7
0
7
14
21
28
35
42
Days Relative to Calving


♦ Control
■ Subclinical
▲ Clinical

Figure 4.

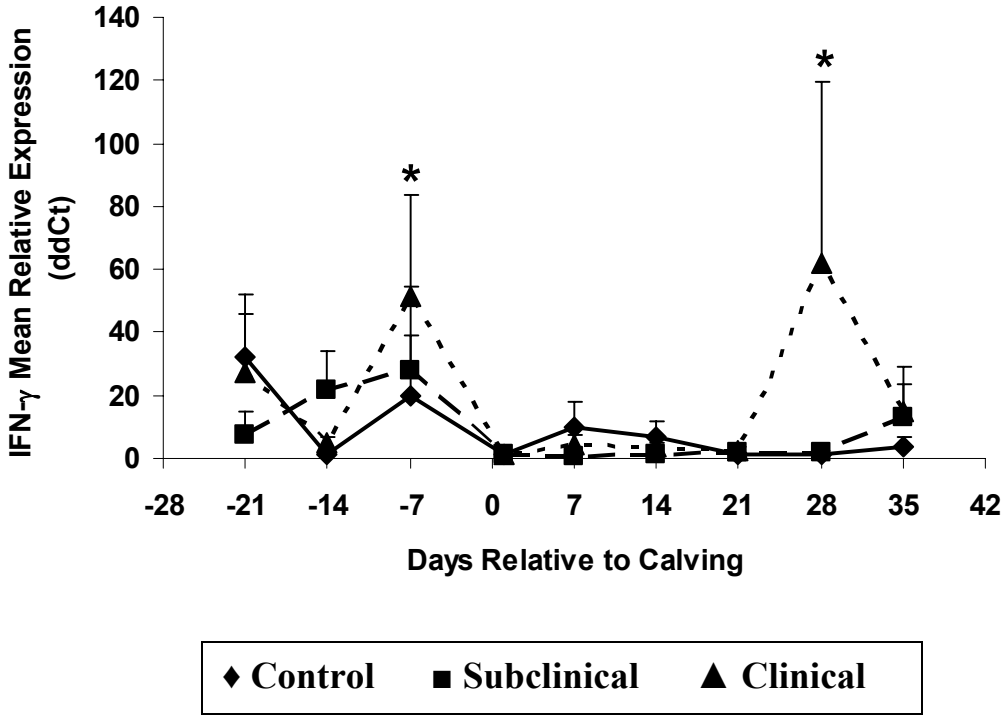
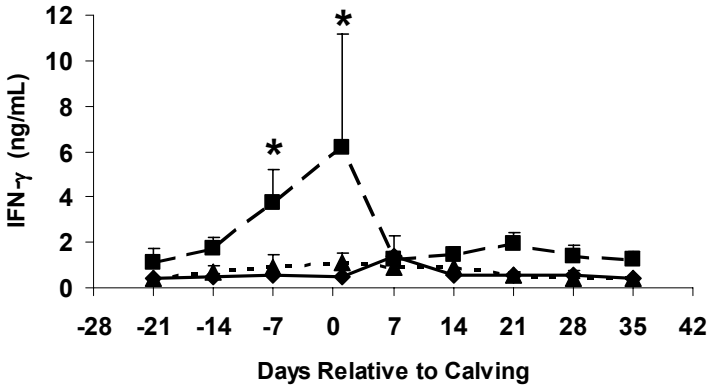
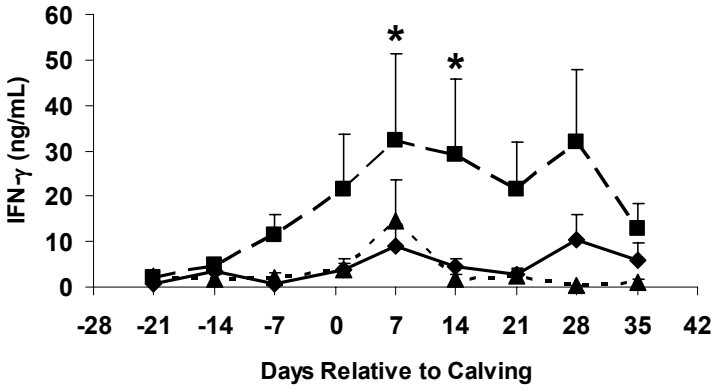

IFN-γ Mean Relative Expression (ddCt)
140
120
100
80
60
40
20
0
-28
-21
-14
-7
0
7
14
21
28
35
42
Days Relative to Calving
*
*
♦ Control
■ Subclinical
▲ Clinical

Figure 5

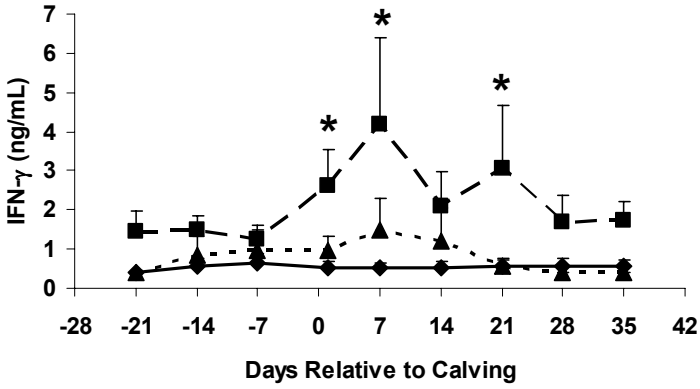
A.


IFN-γ (ng/mL)
12
10
8
6
4
2
0
-28 -21 -14 -7 0 7 14 21 28 35 42
Days Relative to Calving
*
*

B.


IFN-γ (ng/mL)
60
50
40
30
20
10
0
-28 -21 -14 -7 0 7 14 21 28 35 42
Days Relative to Calving
*
*

C.


IFN-γ (ng/mL)
7
6
5
4
3
2
1
0
-28 -21 -14 -7 0 7 14 21 28 35 42
Days Relative to Calving
*
*
*

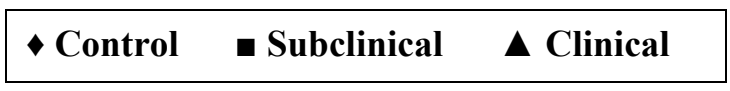
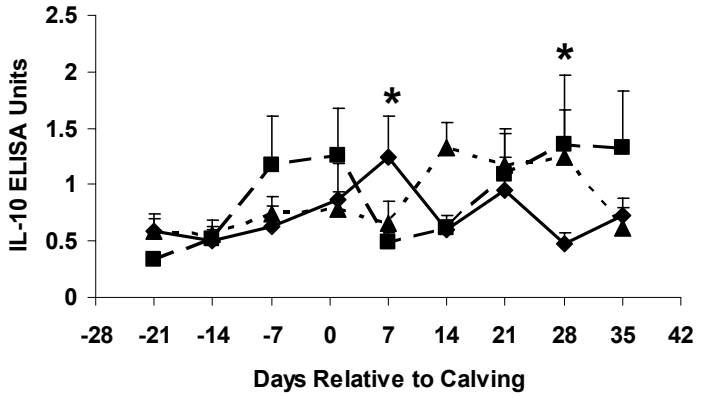
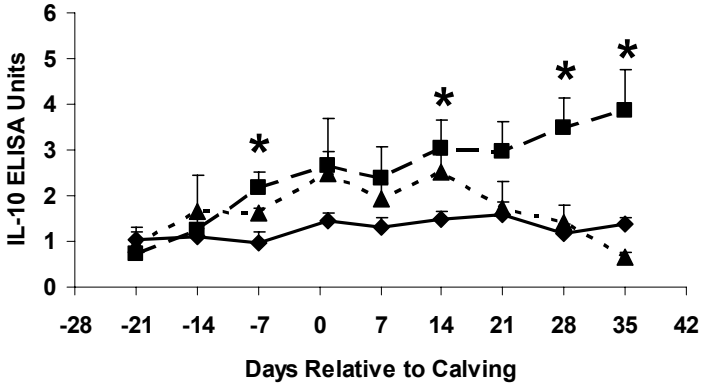

♦ Control
■ Subclinical
▲ Clinical

Figure 6

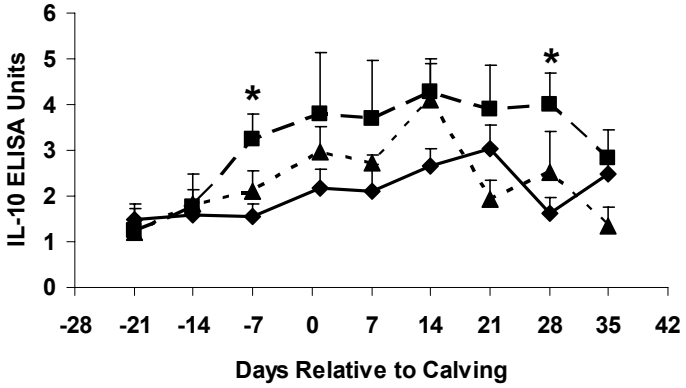
A.


IL-10 ELISA Units
2.5
2
1.5
1
0.5
0
-28 -21 -14 -7 0 7 14 21 28 35 42
Days Relative to Calving
*
*
*

B.


IL-10 ELISA Units
6
5
4
3
2
1
0
-28 -21 -14 -7 0 7 14 21 28 35 42
Days Relative to Calving
*
*
*
*

C.


IL-10 ELISA Units
6
5
4
3
2
1
0
-28 -21 -14 -7 0 7 14 21 28 35 42
Days Relative to Calving
*
*

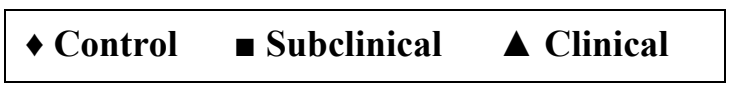
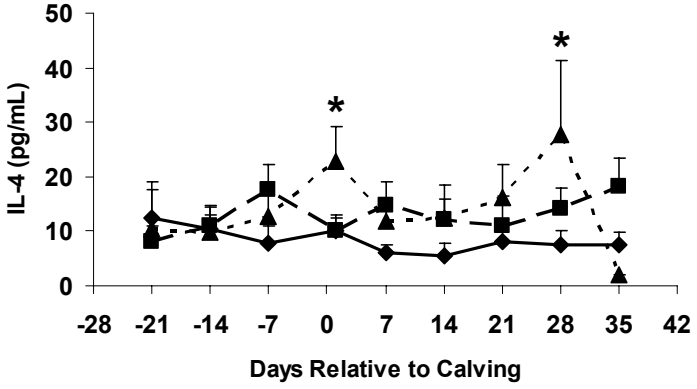
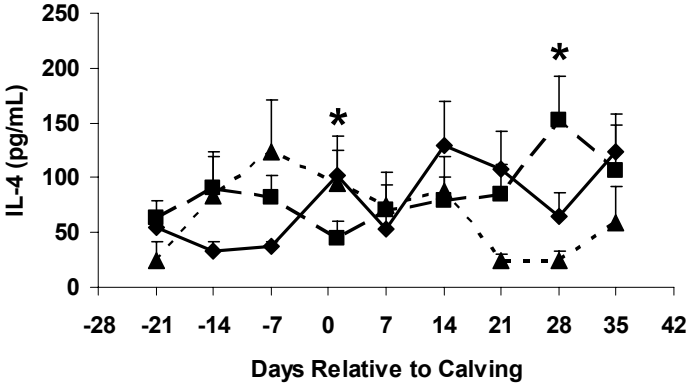

♦ Control
■ Subclinical
▲ Clinical

Figure 7

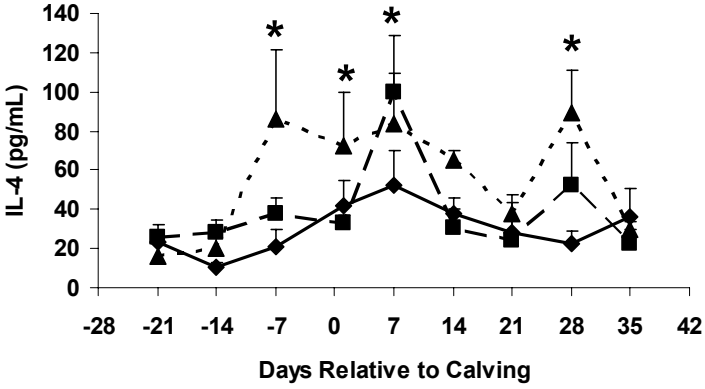
A.


IL-4 (pg/mL)
50
40
30
20
10
0
-28 -21 -14 -7 0 7 14 21 28 35 42
Days Relative to Calving
*
*

B.


IL-4 (pg/mL)
250
200
150
100
50
0
-28 -21 -14 -7 0 7 14 21 28 35 42
Days Relative to Calving
*
*

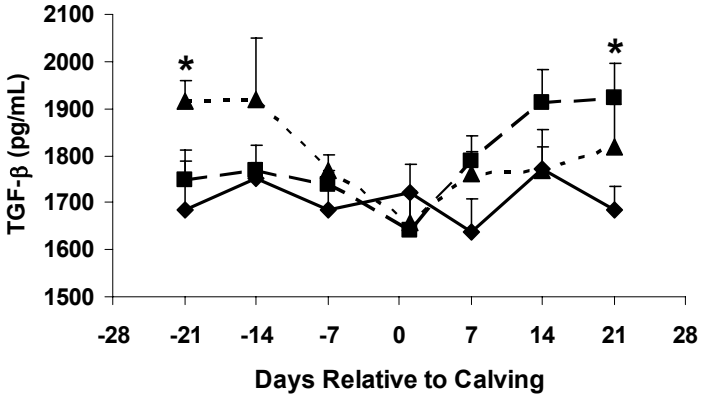
C.


IL-4 (pg/mL)
140
120
100
80
60
40
20
0
-28 -21 -14 -7 0 7 14 21 28 35 42
Days Relative to Calving
*
*
*
*

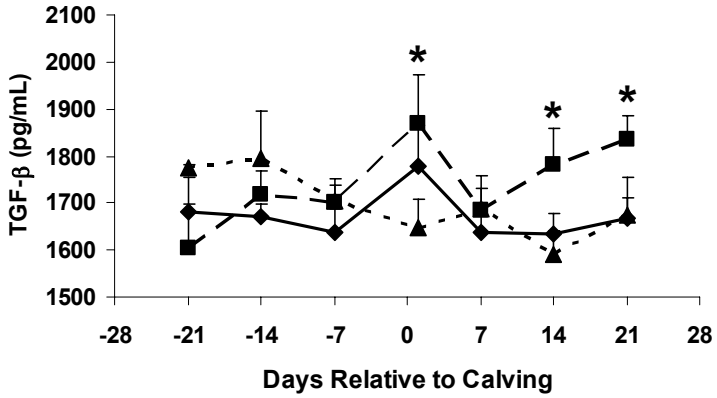

♦ Control
■ Subclinical
▲ Clinical

Figure 8

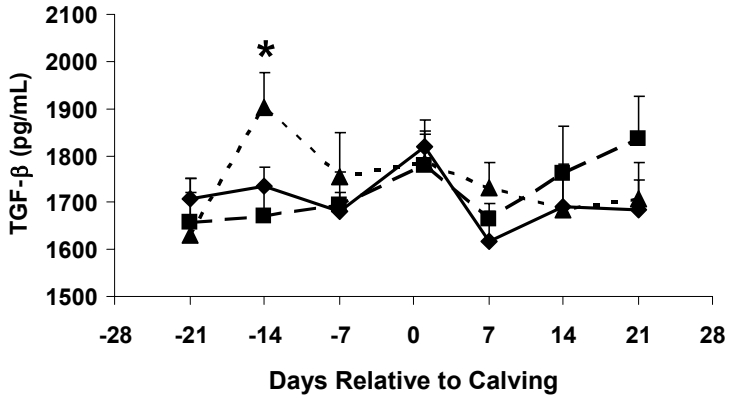
A.


2100
2000
1900
1800
1700
1600
1500
TGF-β (pg/mL)
*
*
-28
-21
-14
-7
0
7
14
21
28
Days Relative to Calving

B.


2100
2000
1900
1800
1700
1600
1500
TGF-β (pg/mL)
*
*
*
-28
-21
-14
-7
0
7
14
21
28
Days Relative to Calving

C.


2100
2000
1900
1800
1700
1600
1500
TGF-β (pg/mL)
*
-28
-21
-14
-7
0
7
14
21
28
Days Relative to Calving

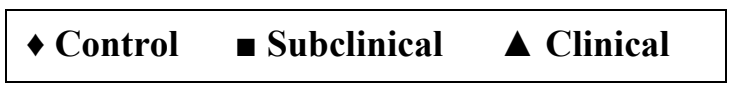
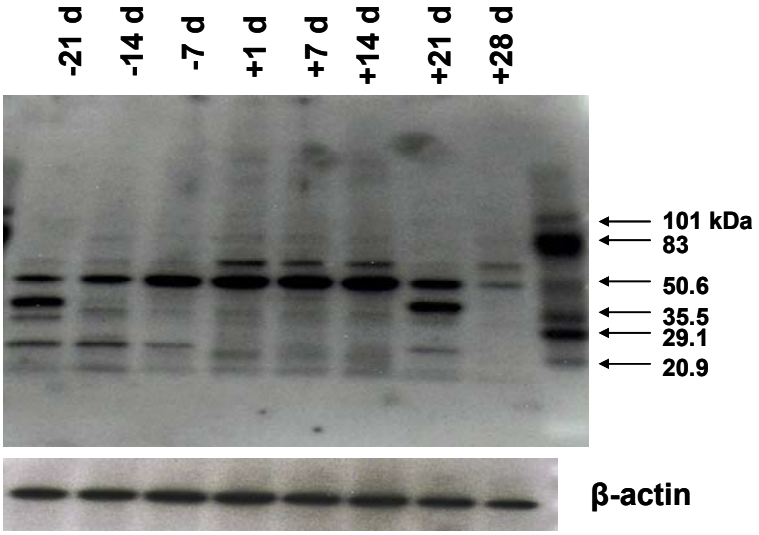
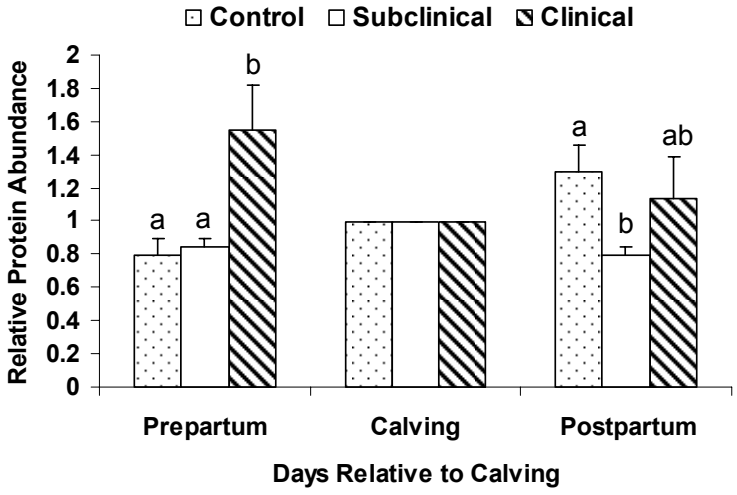

♦ Control
■ Subclinical
▲ Clinical

Figure 9

A.


-21 d
-14 d
-7 d
+1 d
+7 d
+14 d
+21 d
+28 d
101 kDa
83
50.6
35.5
29.1
20.9
β-actin

B.


Control
Subclinical
Clinical
Relative Protein Abundance
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
b
a
b
ab
Prepartum
Calving
Postpartum
Days Relative to Calving

CHAPTER FIVE

OSTEOPONTIN IMMUNOREACTIVITY IN THE ILEUM AND ILEOCECAL LYMPH NODE OF DAIRY COWS NATURALLY INFECTED WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

A short communication to be submitted to *Veterinary Immunology and Immunopathology*

E.L. Karcher[1,4], C.S. Johnson[2], D.C. Beitz[1], J.R. Stabel[3,5]

ABSTRACT

Osteopontin (Opn), a highly acidic glycoprotein, promotes cellular adhesion and recruitment and has been shown to be upregulated in the granulomas of mycobacterial infections. Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is associated with granulomatous enteritis. The objective of this experiment was to identify Opn in the ileum and ileocecal (IC) lymph node of dairy cows naturally infected with MAP and to compare the frequency and intensity of staining between noninfected healthy controls, subclinical and clinical cows. Sections from these three groups were selected from a tissue archive. Immunohistochemical analysis was used to determine the location and expression of Opn. The frequency and intensity of staining were also reported. Confirmation of acid-fast bacilli in the tissue sections was achieved by the Ziehl-Neelsen method. Within the ileal tissue, macrophages, lymphocytes, and plasma cells stained positive for Opn. Clinical cows expressed Opn at a greater frequency in the lamina propria. Control and subclinical cows

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[4]Primary Researcher and author.
[5]Author for correspondence.

did not have areas of granulomatous inflammation, but cells staining for Opn were equally intense for the three groups. Osteopontin expression in the IC node was not affected by MAP infection. Results of this study confirm for the first time the expression of Opn in the ileum and IC node of MAP-infected cattle.

1. INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease, is currently a major economic and welfare issue for dairy producers in the United States. Once infected, animals may remain in the subclinical, or asymptomatic, stage of the disease for several years. The formation of granulomas at the site of MAP infection is critical for the early control of the infection. In subclinical cows, the lesions are predominantly located in the ileal lamina propria and are composed of lymphocytes, plasma cells, and macrophages. As the disease progresses to the clinical stage, animals present with severe weight loss, intermittent diarrhea, and thickening of the intestinal wall. Lesions in clinical cows, consisting primarily of macrophages, are found in the lamina propria, submucosa, and the lymphoid follicles of the Peyer's Patches.

Osteopontin (Opn) is a highly acidic glycoprotein that is produced by activated macrophages (Atkins et al., 1998), activated T-cells (Ashkar et al., 2000), and dendritic cells (Kawamura et al., 2005). The role of Opn in mycobacterial infections is of interest based on its reported ability to promote the production of Th1 cytokines and to enhance host resistance against mycobacteria (Nau et al., 1999; Weber et al., 2002). Stimulating macrophages with Opn resulted in the production of the proinflammatory cytokines, IL-12 and TNF-α (Weber

et al., 2002). The Th1 cytokines, TNF-α and IFN-γ are essential for the production of the protective granulomas in MAP-infected cows (Roach et al., 2002).

Although Opn is constitutively expressed, it is unregulated in inflamed tissues, such as granulomas (Nau et al., 1997). Macrophages interact with Opn through the CD44 receptor and engagement induces chemotaxis and chemoattractant activity (Weber et al., 2002). This interaction is important for the formation of granulomas, as the interaction facilitates the movement of macrophages and lymphocytes from peripheral blood to the tissue.

The objective of this study was to identify Opn in the ileum and ileocecal lymph node (ICN) of dairy cows naturally infected with MAP. This is the first study to report Opn localization in the intestinal tract of MAP-infected cows and differences in Opn expression at the site of infection.

2. MATERIALS AND METHODS

2.1 Samples

The study was designed as a retrospective study in which sections were selected from an archive of tissue samples from previously characterized control, subclinical, and clinical dairy cows. Standard procedure in our laboratory mandates a complete necropsy after death including tissue collection for histological and immunohistochemical examination. Ileum and ileocecal lymph node (ICN) are two tissues routinely collected. While in the herd, animals were routinely evaluated on a quarterly basis to determine infection status, which was monitored by culturing the feces for MAP using standard culture methods (Stabel, 1997). By definition, clinical animals were shedding more than 100 CFU/tube of medium and presented with weight loss and intermittent diarrhea. The noninfected control cows were

characterized by repeated negative fecal cultures performed quarterly over a 3- to 5- year period. In addition, the animals were negative on serologic assays (i.e., production of antibody specific for MAP and IFN-γ) performed during that period. The number of ileal sections included samples from 4 controls, 4 subclinical, and 3 clinical cows. The total number of ICN sections included 5 control, 5 subclinical, and 5 clinical cows. Samples of tissues from all cows were fixed in neutral-buffered 10% zinc-formalin, processed routinely, and embedded in paraffin.

2.2 Immunohistochemistry

Sections were deparaffinized in xylene and rehydrated by an ethanol series (100%, 95%, 70%, distilled water). An antigen retrieval step was performed by boiling slides in citrate buffer (10 μM citric acid, pH 6; Mallinckrodt, Hazelwood, MO) plus 0.05% Tween 20 (Sigma, St. Louis, MO) for 20 min and then incubated at RT for an additional 20 min. Slides were washed in 0.1% saponin (Sigma, St. Louis, MO)-PBS solution for 5 min. Endogenous peroxidases were quenched for 30 min by placing the slides in a 3% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) solution prepared with 0.1% saponin-PBS. Slides were washed for 5 min in 0.1% saponin-PBS before blocking for 30 min in 10% normal goat serum (KPL, Gaithersburg, MD). Rabbit anti-bovine Opn (generously supplied by Dr. Gary Killian; Pennsylvania State University, Almquist Research Centre, University Park, PA) was added to each slide (1:25, diluted in tris/PBS/BSA) and allowed to incubate overnight at 4°C in a humidified chamber. A slide with normal rabbit serum served as a no primary antibody control for each tissue type. The following day, slides were washed in 0.1% saponin-PBS for 5 min and incubated at RT for 30 min with biotinylated goat anti-rabbit IgG (KPL,

Gaithersburg, MD). Following a 5 min wash, slides were treated with streptavidin-horseradish peroxidase (KPL, Gaithersburg, MD) for 30 min. Slides were washed in 0.1% saponin-PBS and incubated in DAB-nickel substrate solution (Vector Laboratories, Burlingame, CA) for 20 min. Washed (3 x 5min) slides were stained with Harris Hematoxylin (Newcomer Supplies, Middleton, WI). Slides were then washed (3 x 5 min) in water, treated with blueing water (ammonium hydroxide) for 1 min, and then washed an additional 3x (1 min/wash). Slides then were dehydrated by an ethanol series (water, 95%, 100%) and xylene, coverslipped with Permount (Fisher Scientific, Pittsburgh, PA), and evaluated for the presence of Opn by light microscopy at 40x magnification.

2.3 Scoring of Immunohistochemistry

For evaluation of Opn in ileal tissue, 10 random fields of the lamina propria were scored for the frequency and intensity of cellular Opn immunoreactivity. For evaluation of the ICN, 10 random fields of the paracortex were scored. The frequency of cell staining was scored on a scale of 0-3 (0= 0%, 1= <25%, 2=25-75%, and 3= >75%. The intensity of the cell staining also was scored on a scale from 0-3 (0= negative, 1= mild intensity, 2= moderate intensity, and 3= intense).

2.4 Ziehl-Neelsen Staining

Sections from the ileum and ICN were cut at 4-6 μm and stained with Ziehl-Neelsen (ZN) by conventional methods. Ten random fields of the lamina propria or the paracortex were scored for ileal tissue and IC node, respectively. Slides were scored on a scale of 0-3

(0= no acid-fast bacilli, 1= small number of bacilli, 2= moderate number of bacilli, 3= large number of bacilli). All slides were read at 40x magnification.

2.5 Statistical Analysis

The data was analyzed using the PROC Mixed analysis of SAS (PROC MIXED in SAS PC Windows Version 9.1.3 software). The model included infection group and frequency of cellular staining or intensity of staining. The values reported are means and standard errors with significance set at $P < 0.05$.

3. RESULTS AND DISCUSSION

We present here the first reported data on the localization of Opn in the ileum and ICN of dairy cows naturally infected with MAP. Infections caused by MAP, the causative agent of Johne's disease, are estimated to cost the US dairy industry upwards of $250 million annually (Ott et al., 1999). MAP is a weakly gram-positive, acid-fast bacillus. Calves most likely become infected with MAP during the first six-months of life (Sweeney et al., 1996). Once infected, cows generally remain in the subclinical, or asymptomatic, stage of the disease for several years until the disease progresses to the clinical state. The disease is associated with chronic granulomatous enteritis.

Osteopontin plays an early role in initiating the innate immune response to mycobacterial infections by promoting cellular adhesion and recruitment of inflammatory cells from the peripheral blood, increasing the number of activated macrophages, and eliciting a Th1 cytokine response. It is produced by activated macrophages (Atkins et al., 1998), activated T-cells (Ashkar et al., 2000), and dendritic cells (Kawamura et al., 2005). In

the current study, macrophages, lymphocytes, and plasma cells in the ileal tissues of all animals stained positive for Opn. This result is in contrast to that in the ICN, in which only lymphocytes stained positive for the subclinical and control groups. Both lymphocytes and macrophages, however, stained positive for Opn in the ICN from clinically infected animals. In all cell types, Opn stained within the cytoplasm. Despite reports of Opn expression by enterocytes and smooth muscle cells, there was no staining of these cell types in either the ileum or ICN.

Progression of MAP-infection to the clinical stage of the disease is characterized by increases in the granulomatous lesions in the lamina propria of the ileum. Macrophages become the predominate immune cell in the lesion and there is a dramatic increase in the bacterial load (Hostetter et al., 2005). This result is consistent with those of the current study in which clinical cows had poorly demarcated areas of severe granulomatous inflammation. These intestinal lesions were composed predominantly of macrophages immunoreactive for Opn (Fig. 1A). Diffusely, the lamina propria in clinical cows was expanded by an abundant number of macrophages. Additionally, the submucosa of clinical cows was multifocally infiltrated and expanded by linear aggregates of macrophages. The staining of the macrophages appeared granular within the cytoplasm of the cell. Clinical cows had a greater percentage of cells staining positive for Opn compared with control cows ($P < 0.01$) (Table 1). Although Opn is expressed constitutively, it is upregulated in inflamed tissues such as granulomas (Nau et al., 1997). Osteopontin interacts with the CD44 receptor on macrophages to induce chemotaxis and chemoattractant activity (Weber et al., 2002). It also induces T-cell chemotaxis and costimulates T-cell proliferation (O'Regan et al., 1999). The ability of Opn to recruit macrophages to sites of inflammation was illustrated when rats that

were injected with recombinant Opn had a 225% increase in the number of macrophages at the injection site compared with the controls (Giachelli et al., 1998). Despite differences in the frequency of staining, there were no differences observed in the intensity of the stained cells (Table 1). Staining by ZN method demonstrated acid-fast bacilli within the cytoplasm of macrophages in the lamina propria of the clinical Johne's cows only (Table 2).

In contrast to the ileum sections, differences in the frequency of cells staining positive for Opn were not observed in the ICN between the different infection groups (Table 2). For both subclinical and control animals, only the lymphocytes were positive for Opn. However, in the clinical animals, both lymphocytes and macrophages were positive (Fig. 1B**)**. Staining by ZN method demonstrated acid-fast bacilli in the paracortex of clinical cows only. Control and subclinical cows were negative for acid-fast bacilli.

In conclusion, the results of this study show for the first time the presence of Opn in ileal and ICN tissue sections from healthy dairy cows and cows naturally infected with MAP. We also showed that Opn was upregulated in the areas of granulomatous inflammation in the ileum of clinical cows. Results presented here are important because they support a role for Opn in the regulation of MAP infection.

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Table 1. Frequency and staining intensity scores of osteopontin immunoreactivity in the ileum[1] and ileocecal[2] lymph node from dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*.

Ileum			
Infection Group	Frequency of Cellular Staining[3]	Staining Intensity[4]	Cell Types
Control	1.43 ± 0.4[a]	1.93 ± 0.3	Lymphocytes Plasma Cells Macrophages
Subclinical	2.25 ± 0.2[ab]	2.04 ± 0.3	Lymphocytes Plasma Cells Macrophages
Clinical	2.93 ± 0.03[b]	1.57 ± 0.03	Macrophages
IC Lymph Node			
Infection Group	Frequency of Cellular Staining	Staining Intensity	Cell Types
Control	1.42 ± 0.6	1.00 ± 0.5	Lymphocytes
Subclinical	2.25 ± 0.4	1.27 ± 0.3	Lymphocytes
Clinical	1.77 ± 0.4	0.64 ± 0.1	Lymphocytes/Macrophages

[1]10 random fields of lamina propria scored.
[2]10 random fields of paracortex scored.
[3]Frequency Score: 0= no cells, 1= <25%, 2= 25-75%, 3= >75%
[4]Staining Intensity: 0= negative, 1= mild, 2= moderate, 3= intense
[a-b]Different superscripts indicate differences ($P < 0.05$).

Table 2. Acid Fast Scoring for ileum[1] and ileocecal[2] lymph node from dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*.

Ileum	
Infection Group	Score[3]
Control	0[a]
Subclinical	0[a]
Clinical	2.73 ± 0.2[b]
IC Lymph Node	
Infection Group	Score
Control	0[a]
Subclinical	0[a]
Clinical	1.97 ± 0.6[b]

[1]10 random fields of lamina propria scored.
[2]10 random fields of paracortex scored.
[3]Score: 0=no acid-fast bacilli, 1= small number, 2= moderate number, 3=large number
[a-b]Different superscripts indicate differences ($P < 0.05$).

List of Figures

Figure 1. Staining of ileum and Ileocecal lymph node from control, subclinical, and clinical MAP-infected cow after DAB staining using a polyclonal antibody to Opn. **A)** The lamina propria contains rare to small numbers of macrophages, lymphocytes, and plasma cells. The arrow highlights intense Opn immunoreactivity in individual lymphocytes. **B)** The paracortex contains multifocal small aggregates of lymphocytes that are immunoreactive for Opn. **C)** The lamina propria contains small numbers of irregular clusters of macrophages, lymphocytes, and plasma cells. The arrow highlights a focus of moderately intense Opn immunoreactivity. **D)** The paracortex contains multifocal aggregates of lymphocytes that are immunoreactive for Opn. **E)** The lamina propria is expanded and the intestinal crypts are separated by poorly demarcated sheets of macrophages. The arrow highlights a focus of granular Opn immunoreactivity. **F)** The paracortex is infiltrated by a poorly defined aggregate of macrophages (arrow) that lacks the granular Opn immunoreactivity seen in the ileum. Note (arrow) the intensely immunoreactive lymphoyctes at the periphery of granulomatous inflammation. (x40) Bar, 50μm.

Figure 2. Section of ileal tissue (A) and IC node (B) from clinical MAP-infected cow stained for acid-fast bacilli using the Ziehl-Neelsen method. (x40) Bar, 50μm.

Figure 1.

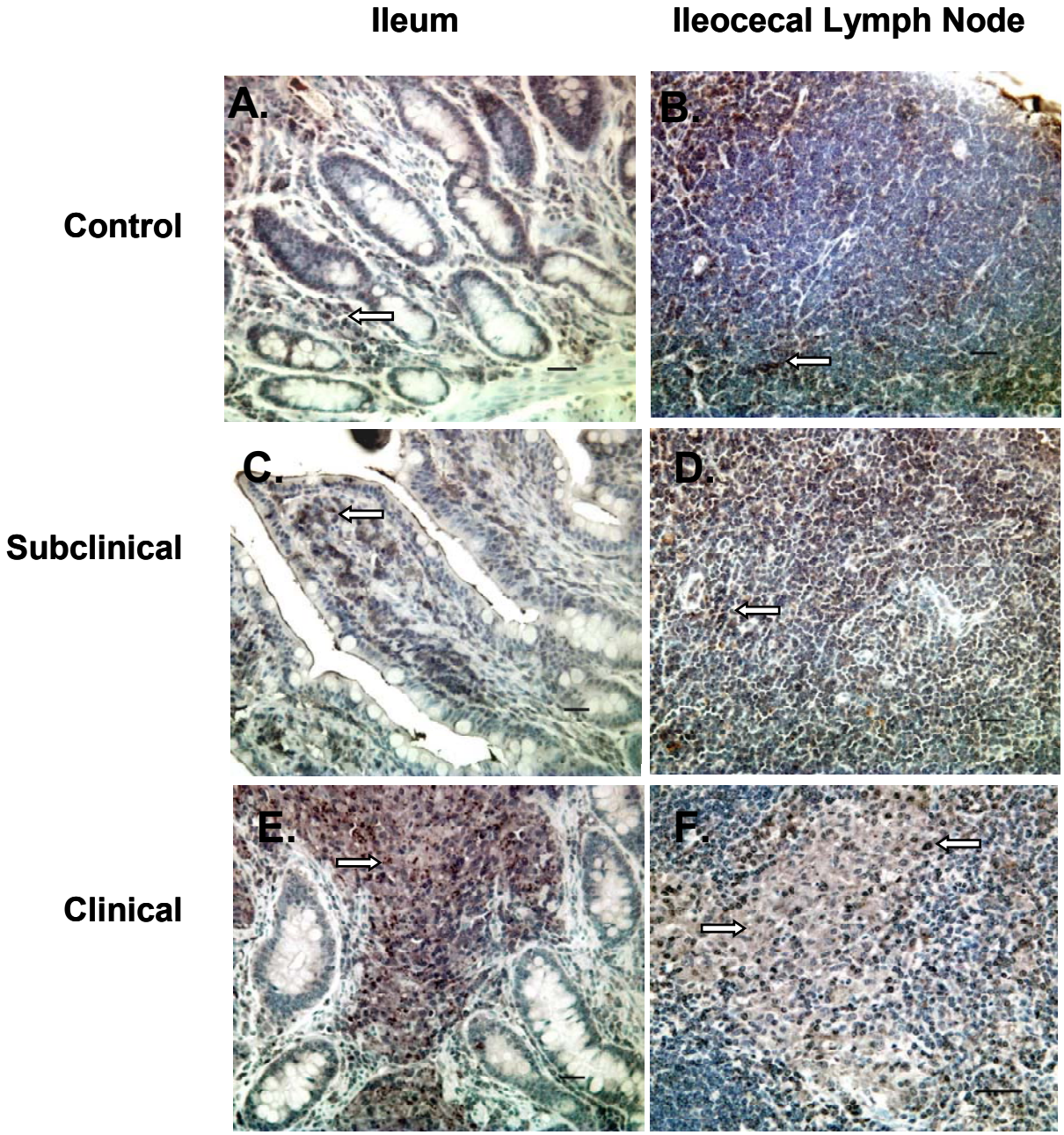
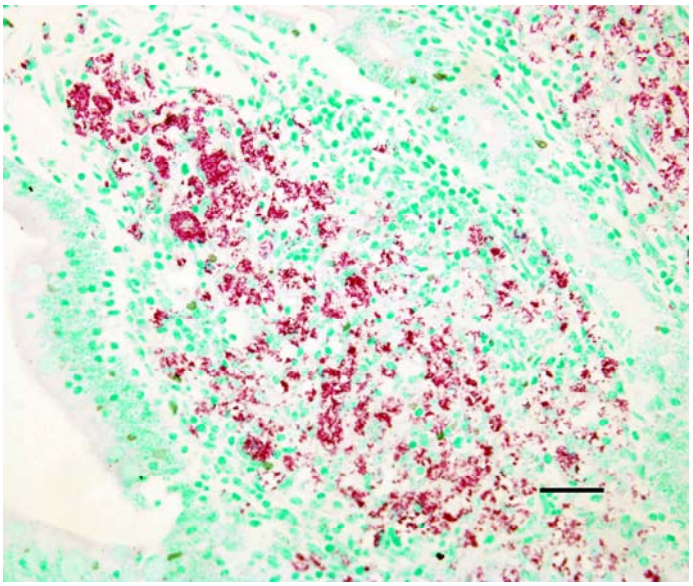

Ileum
Ileocecal Lymph Node
Control
Subclinical
Clinical
A.
B.
C.
D.
E.
F.

Figure 2

A.



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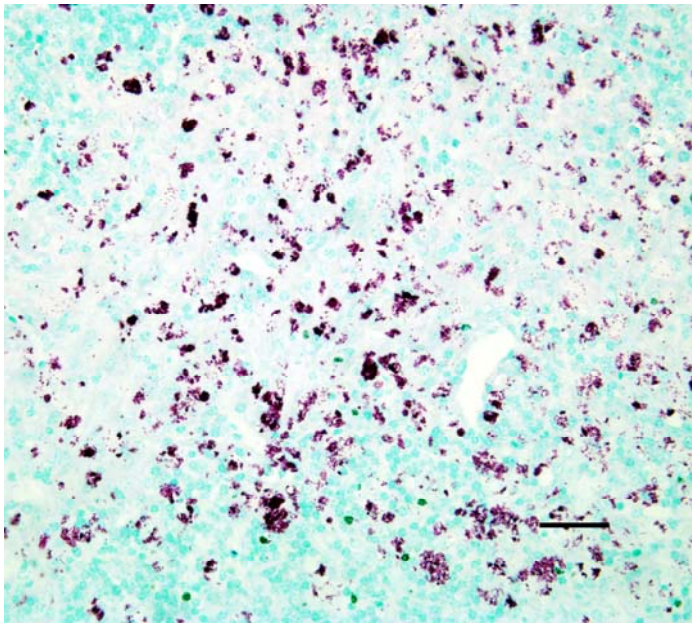

CHAPTER SIX

SUMMARY AND CONCLUSIONS

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease (JD), is estimated to infect more than 22% of US dairy herds and cost the industry $250 million annually. One major period of stress for dairy cows is the periparturient period, and field observations suggest that MAP-infected cows may advance to the clinical stage of the disease during the early post-partum period. Research on what prompts the progression of disease during this time period is lacking. Therefore, the studies presented in this dissertation were guided by the primary goal to gain a better understanding of the host immune response to MAP during the periparturient period. A better understanding of the interaction of these two factors may provide valuable information to the dairy industry. Preventing the incidence of JD is not only an issue of animal welfare but will be economically beneficial to the industry.

Chapter 2 characterized cytokine gene expression and secretion in periparturient dairy cows naturally infected with MAP. Upon ingestion of the bacteria, animals remain in the subclinical, or asymptomatic, stage of the disease for several years. The subclinical stage of the disease is highlighted by a Th1, or cell-mediated, immune response. As the disease progresses to the clinical stage, there is a transition from the Th1 to Th2, or antibody-mediated immune response. In this study, we examined the Th1 cytokines, IFN-γ, TNF-α, and IL-12, the Th2 cytokines, IL-4 and IL-10, and the Th3 cytokine, TGF-β. Despite published reports in the literature describing the Th1/Th2 paradigm in mycobacterial

infections, the results of this study did not support an effect of MAP-infection on cytokine gene expression. One major downfall was the small number of clinical cows and the inability to analyze cytokine gene expression for this group. Expression could not be determined for the clinical cows because due to sampling error, there was only one sample at the reference expression point (+1 day). However, there were effects of parturition on gene expression for control and subclinical cows. Expression of IFN-γ, IL-4, and IL-10 were affected by the periparturient period. We were able to evaluate the secretion of IFN-γ, IL-10, and TGF-β for control, subclinical, and clinical cows. The overall effect of MAP-infection or parturition on cytokine secretion was minimal. Despite the problem of sample size for evaluation of clinical cow gene expression, this study is important because it contributes to a small body of literature examining cytokine gene expression and secretion in periparturient dairy cows with JD. Future studies are required with larger sample sizes to insure sufficient numbers for statistical analysis. Furthermore, during the four-week postpartum period, we did not observe any of the subclinical cows progressing to the clinical stages of the disease. Therefore, it may be important to evaluate early postpartum animals as they progress into lactation.

The objective of the research presented in chapter 3 was to determine the percentages of $CD4^+$, $CD8^+$, and γδ T-cells, B-cells, and monocytes in the peripheral blood of dairy cows naturally infected with MAP during the periparturient period as compared with healthy control cows. In addition, cell populations were delineated further by staining for CD5, a marker for T- and B-cell activation. Parturition has a major impact on the number of T- and B-cells, both components of the adaptive immune system, and the number of monocyte/macrophages, effectors of the innate immune system, in the peripheral blood of

healthy dairy cows. Previous research has described the increased incidence of infectious diseases and declining lymphocyte function during the periparturient period. There are conflicting reports in the literature describing population changes in $CD4^+$, $CD8^+$, γδ T-cells, and B-cells at parturition. It has been reported that B-cells do little to protect the host during the clinical stage of MAP-infection. In this study, fluorescent antibody labeling of lymphocyte subsets and monocytes and flow cytometry was utilized to determine the percentages of specific cell types from three weeks post-partum through four weeks post-partum. Results indicate that in dairy cows the percentages of both lymphocyte subsets and mononuclear cells are modulated by natural infection with MAP and by the periparturient period. In addition, these factors are capable of influencing expression of the activation marker, CD5, on T-cell subpopulations and B-cells. Clinical animals consistently had greater percentages of $CD5^{dim}$ lymphocyte subsets compared with control and subclinical cows. The data presented in this chapter are important because they highlight changes in the immune response of infected cattle during the highly stressful time of parturition. Future research should seek to further characterize the changes in CD5 observed between infected groups.

Osteopontin (Opn), a highly acidic glycoprotein, was examined in chapter 4 because of its known ability to enhance the Th1 response and to increase host resistance to Mycobacterial infections. The objective of this study was to evaluate Opn gene and protein expression in dairy cows naturally infected with MAP during the periparturient period as compared with healthy controls. In addition, Th1 and Th2 cytokines were evaluated for expression and secretion. On the basis of problems presented in chapter 2 with the small number of clinical cows, this study contained seven clinically infected animals. Results revealed that Opn expression and protein abundance in PBMCs isolated from periparturient

cows are modulated by both MAP-infection status and parturition. As hypothesized, nonstimulated PBMCs from subclinically infected cows expressed more Opn than did the control or clinically infected cows. Osteopontin protein was identified in PBMCs at 60, 52, 34, and 27 kDa and was shown to be present at a high intensity in the areas of granulomas formation in clinical cows. This study is extremely important because it presents the first known data investigating Opn gene and protein expression in MAP-infected cows. Further research is required to understand what role Opn may be playing in the progression of JD.

Finally, as a follow-up to the previous study, Opn was further explored in chapter 5. The objective of this short communication was to identify Opn in the ileum and IC node of dairy cows naturally infected with MAP and to compare the frequency and intensity of staining between the different infection groups. In contrast to the findings in chapter 4 that focused on Opn in the peripheral blood, this chapter highlighted Opn localization at the site of MAP infection. Immunohistochemical analysis revealed that, within the ileum, macrophages, lymphocytes, and plasma cells stained positive for Opn. Osteopontin was upregulated in areas of granulomatous inflammation. Ziehl-Neelsen staining for acid-fast bacilli supported increased bacteria in the areas of inflammation. Although Opn was expressed constitutively in the ileocecal lymph node, there were no differences in expression between the infection groups. The results presented in this chapter are the first reported localization of Opn in the ileum and ileocecal lymph node of MAP-infected dairy cows. Additional research is needed to distinguish which subsets of lymphocytes are producing Opn ($CD4^+$, $CD8^+$, or γδ T-cells).

Taken together, data in this dissertation suggest that critical changes during the periparturient period that may alter the ability of the host immune system to respond to MAP.

Future research will be required to gain a better understanding of these critical changes. A study following MAP-infected cows through lactation would be beneficial to further evaluate cytokine profiles and progression of JD. In addition, the Th1 cytokine, IL-18, should be examined. IL-18 has been shown to enhance the production of other Th1 cytokines and enhance the maturation of T-cells. Furthermore, IL-18 has been reported to improve host resistance to other mycobacterial infections.